MOLECULAR AND PHYSIOLOGICAL ANALYSES OF SOLUBLE ACID INVERTASES IN MAIZE

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To my wife, *Juan-Juan Wang*

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MOLECULAR AND PHYSIOLOGICAL ANALYSES OF SOLUBLE ACID INVERTASES IN MAIZE

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The purpose of work presented here was three fold First, the expression of soluble invertase genes in maize (*Zea mays* L) was characterized on a cell and tissue level so that responses to sugars and developmental signals could be interpreted in a physiological context The hypothesis tested was that key sites of altered expression were involved in invertase responses to these stimuli The *Ivr2* soluble acid invertase mRNA was most abundant in cells having a potentially elevated demand for sugars and/or undergoing active expansion. These regions included 1) newly developed embryo and endosperm pericarp, and vascular tissues of ovules and young kernels; and 2) epidermis, new vascular tissues, and elongating cells of the cortex and inner stele of root tips Regulation of *Ivr2* by endogenous signals was suggested by the localized changes induced by pollination Analyses indicated that cytokinins, sugars and interactions between them could alter expression of the *Ivr2* invertases at both tissue and cellular levels

Second, further molecular analyses of these genes [illegible] were isolated, sequenced, and designated as *Ivr1A* and *Ivr2B*. The deduced amino acid sequences were similar to previously identified soluble acid invertases of vascular plants. However, both *Ivr1A* and *Ivr2B* differed from most other invertases in lacking their NDPNG consensus region undivided by introns. Putative sugar- and ABA response elements were evident in 5' promoter regions of *Ivr2A* and *Ivr2B*.

Third, to test hypotheses for molecular mechanisms of gene regulation, GUS reporter genes driven by promoters of the *Ivr1A* and *Ivr2A* soluble acid invertases and their introns were constructed. Analyses of transient expression indicated that up-regulation of soluble acid invertase by the plant growth regulator kinetin was mediated at least partially via transcriptional control in maize. In addition, chimeric gene constructs utilizing the maize *Sh1* first intron significantly increased GUS expression. The presence of the *Ivr2A* intron also enhanced expression of its own gene. This investigation has thus identified the most valuable of the soluble invertase genes, promoters, and introns for use in transgenic strategies for improvement of maize yields.

CHAPTER I
INTRODUCTION

In vascular plants sucrose is the primary form in which photosynthates are transported through phloem to carbohydrate importing organs. As such it is the central and fundamental substrate for growth and development in these plants. When sucrose reaches importing cells it can be cleaved by either invertase or sucrose synthase, the only known plant enzymes that can hydrolyze this disaccharide. Invertase catalyzes an irreversible reaction that hydrolyzes sucrose into fructose and glucose, whereas sucrose synthase catalyzes a reversible reaction that uses UDP to cleave sucrose into fructose and UDP-glucose. Among the three products generated from cleaving sucrose by invertase and sucrose synthase (fructose, glucose and UDP-glucose), only the hexoses can be further metabolized by fructokinase or glucokinase. UDP-glucose follows other paths.

This is important because hexoses such as glucose and fructose (but not UDP-glucose) can initiate one or more paths of sugar signal transduction. Although sucrose itself can be sensed in some instances (Rook et al. 1998, Chiou and Bush 1998), its metabolites seem to be necessary to most cells (reviewed by Koch 1996, Jang and Sheen 1997, Sheen et al. 1999). Evidence from yeast and vascular plants (Jang et al. 1997) indicates that hexose binding to hexokinase can initiate at least one path of sugar signal transduction, and thus modulate the expression of specific sugar responsive genes. For this reason, fructose and glucose can be considered "sensible" sugars whereas UDP

glucose is not. Cleavage of sucrose by invertase can thus generate twice as much sensible sugar as does sucrose synthase. Therefore, invertase activity may have a potentially stronger influence on sugar responsive genes than does that of sucrose synthase.

Sugar-responsive genes can be either up- or down regulated by sugar availability (Table 1.1). Genes encoding enzymes for photosynthesis are commonly up-regulated by depleted sugar supplies and down-regulated by elevated carbohydrates (Sheen 1990). In contrast, genes affecting storage processes and sucrose import by growing organs are often negatively modulated by carbohydrate deprivation and positively regulated by increased sugar availability.

Table 1.1. Sugar modulated genes

Sugar-repressed	**Sugar-enhanced**
Genes for C acquisition e.g., photosynthesis in source leaves	Genes for C use e.g. starch storage in sink tissues
(Stress tolerance?) *Sh1* *Ivr1*	*Sus1* *Ivr2*

Soluble invertase and sucrose synthase are both encoded by gene families that can be subdivided based on their responses to sugar abundance (Koch et al. 1992, Xu et al. 1996). The *Sh1* sucrose synthase and *Ivr1* invertase mRNAs are up-regulated by depletion of sugar supplies, whereas the *Sus1* sucrose synthase and *Ivr1* invertase

mRNAs are up-regulated by elevated carbohydrate availability (Koch et al. 1992 Xu et al. 1996). It is intriguing that both invertase and sucrose synthase not only are sugar responsive, but also produce 100% of the sucrose-derived effectors of sugar signaling (Figure 1-1). Accordingly, invertase and sucrose synthase occupy pivotal positions relative to overall sugar-modulated gene expression in vascular plants.

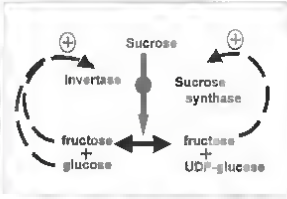


Figure 1-1. The hexose products of sucrose metabolism by invertase and sucrose synthase, can up regulate genes for these same enzymes.

In maize, soluble acid invertase is expressed primarily in rapidly-expanding organs such as tassels, ovules, young kernels, silks, and root tips (Xu et al. 1996, Wu and Koch unpublished data). However, functional roles of these genes remain unclear, so further clues have been sought in more specific localization of their expression by individual cells within these developing organs.

For sucrose synthase, which catalyzes the alternate path of sucrose metabolism, protein has been localized at a cellular level in intact maize root tips (Chen and Chourey

1989 Koch et al 1992) and kernels (Rowland and Chourey 1990 Wittich and Vreugdenhil 1998) Changes in sucrose synthase expression in maize root tips under carbohydrate deprivation favor key cells and tissues (including phloem) which could affect maintenance of essential structures during stresses involving C limitation In contrast, a broad distribution could maximize carbohydrate use throughout importing organs during periods of plentiful carbohydrates (Koch et al 1992) In maize kernels the localized changes in sucrose synthase protein are closely related to sites of maximal starch synthesis during later stages of development (Wittich and Vreugdenhil 1998) Other sites of rapid sucrose use on a cellular level correspond to localized up regulation of sucrose synthase expression (Chen and Chourey 1989 Koch et al 1992)

However despite the importance of both soluble invertase and sucrose synthase to sucrose import, there may be differences in the sites and timing of their expression that could help delineate functional differences between them For this reason *in situ* hybridization techniques have been used here to localize expression of soluble invertase mRNA at the cellular level in maize root tips, kernels and anthers Concurrent analyses of total mRNA levels have also shown that soluble invertases of maize are both sugar and cytokinin responsive (Zeng et al unpublished data) Although several groups have analyzed the promoters of sugar responsive genes, no consensus elements have been identified (Urwin and Jenkings 1997 Hwang et al 1998, Toyofuku et al 1998) Cytokinin-responsive promoters and cis-elements have remained even more elusive. To determine whether any sugar or cytokinin responsive regions were present in soluble invertase promoters genomic clones were obtained and upstream sequences used to drive

chimeric genes with GUS reporters Transient expression assays were used to [illegible] transcriptional responsiveness to sugars and cytokinins by *Ivr1A* and *Ivr2A*

The overall goal of this work was to further clarify the physiological significance and molecular mechanisms regulating soluble invertase genes in maize Specific objective were as follows

1 Test the influence of developmental signals on soluble invertase expression and localization during ovule and young kernel development (Chapter 3)

2 Determine whether sites of soluble invertase expression and cell expansion vary with cytokinin and sugar availability in maize root tips (Chapter 4)

3 Clone and molecularly characterize the genomic DNA of maize *Ivr2A* and *Ivr2B* soluble invertases (Chapter 5)

4 Test the 5 promoters of maize *Ivr1A* and *Ivr2* soluble acid invertase genes in terms of transcriptional regulation that could be exerted by upstream and/or intronic sequences of the soluble invertase genes in maize (Chapter 6)

CHAPTER 2
LITERATURE REVIEW

The first step in sucrose metabolism can be catalyzed by one of only two known enzymes, invertase (sucrase) or sucrose synthase. This cleavage of sucrose comprises a key regulatory point in the transfer of C resources from exporting to importing tissues in vascular plants. Sucrose is not only the primary form of carbohydrate for source-to-sink transport, but effective unloading requires a minimum level of sucrose in the transport stream (Karamdeep et al. 1998). Because sucrose serves as the primary nutrient form transported to carbon-importing organs, the development of sink tissues can be strongly affected by the capacity for sucrose metabolism via invertase and/or sucrose synthase. For decades, the mechanisms of interaction between source and sink have remained a central research focus. An important ultimate goal of these efforts is to increase the import capacity of harvestable sink tissues such as fruits and seeds etc. Although either invertase or sucrose synthase can catalyze the first step of C-metabolism in sink cells, deficiencies of either one can markedly alter development of importing organs.

Maize Soluble Invertase Is Sugar Responsive

Gene responses to sugars can differ markedly. Some genes are up-regulated by plentiful supplies of carbohydrates, whereas others are induced by sugar depletion, i.e.

they respond to "feast or famine" conditions respectively (Koch 1996). Generally, genes encoding enzymes for photosynthesis are up-regulated by depletion of sugar supplies and down regulated by elevated carbohydrate availability. For example, transcriptional activity of maize promoters from photosynthetic genes was repressed by glucose and sucrose (Sheen 1990). In contrast, genes affecting storage processes and the strength of sink organs are negatively modulated by carbohydrate deprivation and positively regulated by increased sugar levels. "Feast or famine" conditions specifically up-regulate respective subgroups of gene families encoding both invertase and sucrose synthase. Depletion of sugar supplies increases expression of *Ivr1* invertase and *Sh1* sucrose synthase, whereas elevated carbohydrate availability up-regulates the *Ivr2* invertase and *Sus1* sucrose synthase (Koch et al. 1992, Xu et al. 1996).

Invertase Is Important for Sugar Signaling

Not only are invertase and sucrose synthase genes sugar-modulated, but their activity may also affect hexose-based sugar sensing systems in vascular plants. These two enzymes thus occupy a pivotal position in the overall process of carbon allocation and mobilization. The detailed molecular mechanisms of sugar sensing and sugar mediated signal transduction pathways have yet to be defined. However, recent studies suggested that hexokinase might be a key factor in higher plants for sugar sensing in a manner similar to that in yeast. By using transgenic *Arabidopsis* either under- or over-expressing hexokinase, Jang et al. (1997) obtained evidence that this enzyme may act as a sugar sensor early in at least one path of sugar signal transduction. The actual mechanism of signal initiation remains unknown, but may involve conformational change

of the enzyme-product complex, its subcellular localization and/or its relationship to membranes or other proteins (Graham 1996 Koch et al 2000) This signal transduction pathway typically up-regulates genes that respond to carbohydrate deprivation (e g photosynthetic genes) but also affects some genes induced by sugars (e.g nitrate reductase)

The influence of hexose on this and other hexose-based sugar-sensing systems puts invertase in an important position. Products of the invertase reaction contribute two-fold more "sensible substrates (glucose + fructose) for generation of sugar signals than does sucrose synthase (fructose [+UDP glucose]) There is no currently known mechanism for sensing UDPG the other sucrose synthase product.

A sucrose non fermenting-1-(SNF1)-related protein kinase gene (*PKIN1*) is central to the protein kinase cascade mechanism for sugar signal transduction in yeast (reviewed in Koch 1997 Koch et al 2000) The possible role of an orthologous gene in plants has recently been studied in potato (Purcell et al. 1998) The SNF1-related protein kinase activity was markedly reduced by stable transformation of an antisense SNF1-related protein kinase gene (*PKIN1*) sequence driven by either a patatin (tuber-specific) or a *ST LS1* (leaf and stem-specific) gene promoter Both resulted in a dramatic reduction of sucrose synthase at transcription and enzyme activity levels, as well as blocking the sucrose-inducibility of this gene Expression of hexokinase and invertase genes, however remained similar to that in wild type plants These results indicated that sucrose synthase and invertase may respond to sugar signals by different mechanisms

Maize Soluble Invertase Is Up-Regulated by both Cytokinins and ABA

Increasing evidence has indicated that the extent of morphogenic responses to sugar levels and sugar signaling may be comparable to changes induced by shifts in hormone balance (Koch 1996 Chamere and Hahne 1998) Interestingly invertase but not sucrose synthase can be up-regulated by cytokinins suggesting that cytokinins may have a positive effect on initiating hexose based sugar signals by altering the invertase/sucrose synthase ratio (Zeng et al unpublished data) Evidence also indicates that cytokinin enhancement of invertase expression may be involved in the initial events of reproductive development and meristem formation In addition to sucrose, cytokinins are also transported to newly-growing organs Isopentenyl transferase (IPT) is considered to be a rate limiting enzyme for cytokinin synthesis (Medford et al 1989) In transgenic tobacco plants which were altered to express an IPT gene driven by a senescence-specific SAG12 promoter both glucose and fructose levels increased more rapidly with leaf age than they did in wild type plants (Wingler et al 1998) This would be consistent with up-regulation of sucrose cleavage activity most likely via invertase rather than sucrose synthase The observation that both *Ivr1* and *Ivr2* invertase can be up-regulated by cytokinins, hence raising the invertase/sucrose synthase ratio suggests that this may provide one mechanism contributing to modification of local sugar sensing by phytohormones

The means by which cytokinins regulate gene expression appears to be largely post-transcriptional (Crowell 1994 Silver et al 1996, Downes and Crowell 1998) However cytokinin responsive genes have not yet been identified at a transcriptional level Possible exceptions are a strongly responsive maize gene currently under

investigation by T Sugiyama and Co-workers in Nagoya, Japan (personal communication) and the soluble invertase genes examined here

Soluble invertase genes in maize can be up-regulated at the mRNA and enzyme activity levels by both cytokinin and ABA (Zeng et al unpublished data) To date no other enzymes have been reported for which both cytokinin and ABA have a positive effect. RNA gel blot analyses and enzyme assays have shown that either cytokinin or ABA alone can up-regulate maize invertases by 50% to 100% However cytokinin and ABA together result in as much as 2 to 3- fold increases in levels of both *Ivr1* and *Ivr2* mRNAs as well as total enzyme activity In contrast, numerous studies of other enzymes, or genes have indicated opposing effects of cytokinin and ABA One such example is evident in the work of MooreGordon et al (1998) who found that increases in the endogenous cytokinin/ABA ratios also delayed seed coat senescence and fruit development in *Hass avocado* In lupine cytokinin can promote the greening of etiolated seedlings and plastid biogenesis (Kusnetsov et al 1998) as well as increase growth of buds and branches (Emery et al 1998) In contrast, ABA showed opposite effects It is unclear why invertase in particular is positively regulated by both cytokinin and ABA, or what the physiological significance of this dual response may be Under stress, this combined response may contribute to improved osmotic status, storage of vacuolar sugar reserves and/or enhancement of hexose-based signals to other genes (many of which are considered beneficial under stress) The observation that ABA inhibits maize endosperm cell division but not elongation (Mambelli and Setter 1998) corresponds well to *in situ* results indicating that invertase mRNA is highly localized in elongation zones of maize root tips (Wu and Koch unpublished data).

Invertase and Sucrose Synthase Activities Are closely Related to Rapid Growth

There remains an obvious correlation between rapid growth and high activity of invertase and sucrose synthase (Morris and Arthur 1984 Sung et al 1988) Defects in either of these two enzymes can potentially impair the development of sugar importing organs The maize *sh1* mutant produces markedly smaller kernels than does wild type (Chourey and Nelson 1976) This may be due either to defective starch synthesis or reduced cell wall biosynthesis in these mutants because of their altered sucrose metabolism Reduced expression of cell wall and soluble invertases in the maize mutant, *miniature* are also associated with profoundly reduced kernel size (Miller and Chourey 1992)

Studies comparing maize wild type to *sh1* mutant plants have shown that sucrose synthase is expressed predominantly in kernels during the phase of rapid starch biosynthesis (Chourey et al. 1991) and the resulting phenotype indicates a physiologically important function for the *Sh1* sucrose synthase at this stage Sucrose synthase localization was also examined throughout development of maize kernels (Wittich and Vreugdenhil 1992) Activity was low in kernels until 5 DAP After this time from 10 to 28 DAP activity of sucrose synthase shifted gradually from the apical to the basal regions of the endosperm, correlating with changes in localized starch synthesis during kernel development. Sucrose synthase has also been localized in phloem companion cells of sucrose importing and exporting organs in maize (Nolte and Koch 1993) One possible role for sucrose synthase is to provide energy for transport processes and UDP-glucose for synthesis of callose and/or cellulose (plus other cell wall

constituents). In tomato, sucrose synthase seems to be essential to sink metabolism and starch storage during early fruit growth (D Aoust et al. 1999), whereas the activity of soluble acid invertase determines the hexose levels later during maturation of storage tissues (Damell et al. 1994; Klann et al. 1996). How sucrose metabolism via sucrose synthase vs soluble invertase is coordinated remains unclear.

Soluble Invertase Is Important for Initial Development of Maize Kernels

Soluble acid invertase appears to be closely associated with the initial growth of new, rapidly-expanding sink tissues in a number of different species (Morris and Arthur 1984; Sung et al. 1988; Rabe and Krutschera 1998; Lo Bianco et al. 1999). Data from previous work with maize also indicated that soluble acid invertase may be central to early development of expanding kernels (Xu et al. 1996; Zinselmeier et al. 1999). Sucrose synthase and the insoluble cell wall invertases appear to be more important later in kernel development (Shannon and Dougherty 1972; Chourey and Nelson 1976; Miller and Chourey 1992). Enzyme activity of the extracellular invertases is low during the initial 2 or 3 days after pollination and peaks sometime after the first 10 days of kernel growth. However, soluble acid invertase mRNAs are primarily expressed at early stages of kernel development, especially within the first 6 days after pollination (Xu et al. 1996; Wu and Koch unpublished data). Messenger RNAs of soluble acid invertase are also detectable in maize ovules 2 days before silk emergence (Wu and Koch unpublished data). In addition, a miniature maize mutant with reduced levels of mRNAs and enzyme activity for both soluble and insoluble invertases begins to show a decreased number of endosperm cells at 6 days after pollination and ceased kernel development at about 12

days after pollination (Zhou et al. 1997). Soluble invertase is found primarily inside the vacuoles and in some instances can be present in the cell wall (Sturm 1999). Its expression pattern at a cell level, however remains unknown during early kernel development. Vacuolar invertase may contribute to both sugar metabolism and cell elongation by cleavage of sucrose inside the vacuole, this in turn leading to altered osmotic pressure inside the cell.

Sink Strength Can Be Enhanced by Transgenic Invertase

The interaction between importing and exporting organs can be altered in transgenic plants through manipulation of genes encoding key enzymes of sugar metabolism. In *Vicia narbonensis* expressing a yeast invertase gene targeted to the mature embryo, Weber et al. (1998) found that sucrose decreased and hexose markedly increased in the importing cotyledons. After feeding ^{14}C sucrose, transgenic cotyledons (with elevated invertase) also partitioned more carbon toward protein vs. starch when compared to wild type. Export of sucrose can be blocked in transgenic tomato (Dickinson et al. 1991), tobacco, and *Arabidopsis* (Stitt et al. 1990, Von Schaewen et al. 1990) by expressing yeast invertase in the apoplastic spaces of leaves. Apoplastic expression of an external invertase in carbon-exporting tissues effectively cleaves sucrose that might otherwise have been transported to carbon importing tissues via phloem. This results in accumulation of hexoses, and their consequent down regulation of genes for Calvin cycle enzymes. Photosynthetic rates then drop. Sink strength can also be differentially manipulated by expressing yeast invertase in either cytosol or apoplast of potato tubers (Sonnewald et al. 1997). Cytosolic targeting of yeast invertase gave rise to

increased tuber number and reduced tuber size whereas apoplastic localization resulted in decreased tuber number and enlarged tuber size (Sonnewald et al 1997) Over expression of both yeast invertase and bacterial glucokinase in potato tubers can significantly reduce both sucrose and starch levels, although these transformations increase levels of glucose and other intermediates of glycolysis by 2 to 3 fold (Trethewey et al 1998) (hypoxia may be induced by glycolytic overload in this instance) Overall these results are consistent with the capacity for elevated sugar levels to alter expression of genes via hexose-based sensing systems (Koch 1996 Koch 2000)

Non Consensus *cis* Elements Have Been Defined in Promoters of Sugar Responsive Genes

Mechanisms underlying such instances of sugar modulated gene expression have been pursued at the transcriptional level but remain unclear A number of attempts have been made to define the promoter elements and transcriptional factors that mediate sugar responses Sheen (1990) found sugar repressive elements in several photosynthetic genes upstream of their TATA boxes but no consensus sequences could be identified among them. In addition there appeared to be no similarities to conserved sequences from other non-plant organisms By 5 deletion analysis a 16-bp sequence, termed IMH2, in the promoter of malate synthase from cucumber was found to be a sugar responsive *cis*-element (Sarah et al 1996) Evidence also indicated that in the *rbcS2* gene promoter of *Phaseolus vulgaris* the -205 to 187 region is essential for a strong response to sucrose-repressible expression (Urwin and Jenkins 1997) This region contains a G-box (CACGTG) located at 205 to 200 and two other sequences resembling the SURE

("SUcrose Responsive Element in potato genes, Grierson et al 1994) and the CholRE ("Carbohydrate [Cho] Responsive Element" in mammalian genes Kaytor et al 1997) Two other groups have independently investigated sugar responsive *cis*-elements in an amylase *Amy3D* promoter Hwang et al (1998) reported that three elements are required for maximum effect on *Amy3D* gene expression under conditions of sugar starvation. These are the "Amylase element" (TATCCAT) the "CGACG element" and a 'G box related element (CTACGTGGCCA) In addition Toyofuku et al (1998) found that a 50-bp nucleotide sequence in the promoter region from 172 to −123 is essential for induction of *Amy3D* by sugar depletion. They further characterized a G motif (TACGTA) and a TATCCAT/C motif that were sugar repressible Thus far these studies have not identified consensus sequences for *cis* elements of sugar inducible responses although numerous studies have indicated that an ACT core may be central to this function (Ishiguro and Nakamura 1992, Grierson et al 1994 Kaytor et al 1997) Molecular characterization of the sugar-responsive invertases will contribute further information on this question as well as that of transcriptional vs. translational control (in conjunction with other studies) Also, single-cell responses to sugars are integrated into a whole plant context by their interface with phytohormones, and the following work will determine the extent to which this occurs at a transcriptional level

CHAPTER 3
DEVELOPMENTAL SIGNALS ALTER EXPRESSION OF SOLUBLE INVERTASE GENES IN SPECIFIC CELLS DURING OVULE AND EARLY KERNEL GROWTH

Introduction

The utilization of sucrose can be initiated only via invertase or sucrose synthase In vascular plants different isoforms of invertases are found in different cellular compartments (Avigad 1982 Hawker 1985) Isoforms localized in the vacuole and/or apoplast have an acidic pH optimum whereas those found in the cytoplasm have a neutral pH optimum In addition acid invertases can be either soluble or insoluble depending on the degree to which they are bound to cell wall constituents. Soluble acid invertases can be loosely bound in the cell wall but typically are localized in vacuoles of sugar importing cells (Avigad 1982) In this compartment, mobilization of temporarily stored sucrose by soluble invertases can contribute to the turgor needed for cell expansion. In contrast insoluble acid invertases (cell wall bound) have been suggested to hydrolyze sucrose in the extracellular space near sites of phloem unloading in some tissues such as maturing corn kernels (Shannon and Dougherty 1972, Shannon et al 1993) and sugar cane stems during their storage phase (Glasziou and Gayler 1972) At these sites and stages of development, insoluble invertase is hypothesized to contribute to formation of a sucrose concentration gradient between phloem and apoplast, and thus facilitate

movement of sucrose into carbohydrate-importing tissues (Doehlert 1986, Turgeon 1989).

The activity of soluble invertase is frequently associated with rapid growth of newly initiated and/or expanding sink tissues (Avigad 1982, Morris and Arthur 1984, Schaffer et al. 1987). In maize kernels, soluble invertase is the first enzyme of sucrose metabolism to be up-regulated in response to pollination. Soluble acid invertase is predominantly expressed during the first 6 days after pollination (Xu et al. 1996). Messenger RNAs of soluble acid invertases are also detectable in maize ovules 2 days before silk emergence. Cell wall invertase activity is relatively low prior to pollination and for the first few days after fertilization. These insoluble invertases are important during later stages of kernel development, particularly during kernel fill, and their activity peaks at varying points later than 10 days post pollination.

Maize grain yield is often correlated with kernel number (Otegui and Bonhomme 1998, Habben and Helentjaris unpublished data) and successful initial development of the endosperm and embryo is fundamental to kernel set (Maddonni et al. 1998, Scanlon and Myers 1998). The presilking environment can have a pronounced influence on the potential number of kernels, with postsilking conditions also affecting the final number of fixed kernels (Otegui and Bonhomme 1998, Scanlon and Myers 1998). The activity of soluble acid invertase changes dramatically in wild type ovules and kernels during the last 2 days before silking and the first 6 days after pollination, respectively (Wu and Koch unpublished data, Xu et al. 1996). The *miniature* maize mutant, which is deficient in both soluble and cell wall invertase, has a decreased number of endosperm cells (Zhou et al. 1997) and has only 20 to 30% of the normal seed weight (Cheng and Chourey 1999).

Recent studies have shown that the *Incw* [illegible] is closely associated with a QTL (quantitative trait locus) for water stress induced (Pelleschi et al. 1999, Prioul et al. 1999).

Invertase has the potential to enhance that percentage of imported carbon which is sensed by hexose-based sugar signaling systems (Kawabata and Sakiyama 1998, Isla et al. 1998, Lingle 1999). These "sensible" hexoses can in turn modulate numerous sugar responsive genes in various tissues (Koch 1996, Jang et al. 1997, Sheen et al. 1999). Interestingly, these sugar signals can regulate invertase itself (Figure 1-1). Soluble acid invertase can also be up-regulated by both cytokinins and ABA (Zeng et al. unpublished data). Although initial kernel development is promoted by cytokinin accumulation (Banowetz et al. 1999), exogenously applied ABA inhibits maize endosperm cell division and endoreduplication (Mambelli and Setter 1998). The up-regulation of soluble invertase activity by both cytokinins and ABA together could at least partially offset the negative impact of ABA on early kernel abortion.

The expression pattern of soluble acid invertase at a cellular level could clarify its role during early stages of kernel development, but this information has not been previously available. Soluble invertase is widely associated with expansion of newly growing tissues, where it is found primarily inside the vacuole and in other cell compartments such as the cell wall (Sturm and Chrispeels 1990, Klann et al. 1992, Arai et al. 1992, Ramloch-Lorenz et al. 1993, Schwebel-Dugue et al. 1994). Vacuolar invertase may contribute to both sugar metabolism and cell elongation by cleaving sucrose inside the vacuole (reviewed by Avigad 1982, Sturm 1999). This in turn can alter cell osmotic pressure. As noted above, an additional and possibly more important

role may be that of producing hexoses for amplification of sugar signals from imported sucrose. The vacuolar locale for these processes also introduces a potential for temporal regulation of both osmotic responses and sugar signals. Soluble invertase may thus occupy a central position for carbon metabolism, cell expansion, and generation of sugar signals during early kernel development when the extent of seed set is determined.

Materials and Methods

Plant Materials

Ovules and kernels of the maize (*Zea Mays* L.) hybrid Nk508 were used for these experiments except where tissues from the W22 inbred were examined for comparison. Plants were grown under field conditions (North Florida Spring growing season, April - July) or in greenhouses for analysis of developmental changes in soluble acid invertase expression and the influence of pollination treatments. Ovules were pollinated one day after silking. Samples of ovules and young kernels were harvested at daily intervals and were immediately frozen in liquid N_2 or fixed in cold FAA (10% formalin: 5% acetic acid, 45% ethanol).

RNA Isolation and Gel Blot Analysis

Frozen samples were ground into fine powder in liquid N_2. Total RNA was extracted as per Koch et al. (1992) and quantified spectrophotometrically (Sambrook et

al. 1989 Maliga et al. 1995) Ten micrograms of total RNA were separated by electrophoresis in 1% agarose gels containing formaldehyde, transferred to a nylon membrane and fixed by 8 min of UV radiation. Membranes were hybridized for ca. 16 h at 65°C with maize *hvr2* cDNA (Xu et al. 1996) radiolabeled via random primers (RTS RadPrime DNA Labeling System, Life Technologies, Gaithersburg, MD) After stringent rinsing of the membrane, relative abundance of mRNA was quantified using a phosphor imager (Molecular Dynamics, Sunnyvale CA). In some instances mRNA was also visualized by exposure to X-ray film with intensifying screens at -80°C

Probes for *in situ* Hybridization

A 576-bp NcoI – NotI fragment of the *hvr2* cDNA (the NcoI and NotI sites respectively lie 64 and 640 bp downstream from the 5' end of this cDNA and approximately 130 bp downstream of the conserved NDPNG consensus sequence common to functional invertases defined thus far) was selected based on its minimal homology to *hvr1*. This fragment was subcloned into the NotI and HincII sites of a Bluescript II SK plasmid which has T3 and T7 promoters at each side of its polylinker. DNA templates were linearized with either NotI or EcoRI for *in vitro* transcription, and driven by T3 or T7 promoters respectively. Sense and antisense RNA probes were synthesized with digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis, IN) according to manufacturer's instructions. Probes were hydrolyzed with carbonate buffer (pH 10.2) at 60°C for 22 min to yield an average size of ca. 200 bp. For each slide, 25 ng of RNA probe was applied for *in situ* hybridization.

Tissue Preparation and *in situ* Hybridization

Samples were immediately fixed in FAA (10% formalin, 5% acetic acid, 45% ethanol) at 4°C overnight. Vacuum was applied during the first 2 hours of fixation. Fixed plant tissues were dehydrated in a series of increasing concentrations of ethanol and Histo-Clear (National Diagnostics, Atlanta, GA) before being embedded into paraplast. Embedded tissues were cut into 10 µm sections with a microtome and mounted on ProbeOn Plus microscope slides (Fisher Scientific, Pittsburgh, PA). *In situ* hybridization procedures were conducted as per Jackson *et al* (1994). Tissue sections were pre-treated with 0.2 M HCl for 20 min, 1 µg/ml proteinase K for 30 min, 4% paraformaldehyde for 10 min, and 0.5% (v/v) acetic anhydride for 10 min before being probed with labeled sense or antisense RNAs.

Hybridization at 55°C for 12 h was followed by 2 h of rinsing in 0.2 x SSC at 50°C. Hybridized sections were treated with 20 µg/ml RNase A at 37°C for 30 min followed by another hour of stringent rinsing in 0.2 x SSC at 50°C. For immunological detection of hybridized RNA probes, sections were incubated with alkaline phosphatase conjugated anti-dig antibody (diluted 1:1000) for 2 hours at room temperature after incubation for 45 min in 1% blocking agent (Boehringer Mannheim, Indianapolis, IN) and another 45 min in 1% BSA. Visible product was generated from alkaline phosphatase activity by incubating the sections with 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 50 mM $MgCl_2$ containing a 2% (v/v) mixture of NBT and BCIP (Boehringer Mannheim, Indianapolis, IN) for 1 to 3 days at room temperature. Sections were

dehydrated with increasing concentration of ethanol and histoclear before mounting with Permount (Fisher Scientific, Pittsburgh, PA).

Results

Expression of the *Ivr2* Soluble Invertase in Ovules and Young Kernels

The *Ivr2* soluble invertase mRNA levels responded strongly to change in development (Figure 3-1) rising markedly in ovules immediately before and after pollination. In both the NK508 hybrid and the W22 inbred, *Ivr2* message abundance was low in ovules prior to 4 days before silking. However *Ivr2* mRNA levels rose rapidly and peaked 2 to 4 days after pollination. The *Ivr2* message abundance dropped abruptly after this period and was not detectable in kernels 10 or more days post pollination.

In contrast, when pollination was withheld from receptive ovules, levels of *Ivr2* mRNA remained stable for at least 3 days before slowly decreasing over an extended period (Figure 3-2). The *Ivr2* mRNA in these unpollinated ovules was low after 12 (W22) to 14 (NK508) days post-silking whereas the same messenger RNA was undetectable in the corresponding kernels later than 10 (W22) to 13 (NK508) days post-pollination. Pollination/fertilization signals thus stimulated a rapid rise and subsequent drop in *Ivr2* expression, neither of which were evident in non-pollinated controls. Changes of total soluble invertase activity followed a pattern comparable to that of *Ivr2* mRNA (Figure 3-3).

Tissue-level expression of *Ivr2* soluble acid invertase in ovules and kernels of maize NK 508 was localized via *in situ* hybridization techniques (Figure 3-4). In ovules 1 and 2 days before silking *Ivr2* mRNA was primarily expressed in or near vascular bundles and in cells around the egg sac. Both sites are consistent with a role for elevated invertase activity in facilitating sucrose import.

Localization of *Ivr2* mRNA via *in situ* hybridization revealed that in kernels 2 days after pollination, *Ivr2* soluble acid invertase was specifically expressed in pericarp and vascular tissues (Figure 3-5). In kernels at this stage of development, cells of the pericarp and nucellus are the predominant sink tissues (Kiesselbach 1949). Transcript levels were greatest in tissues undergoing rapid growth and/or cell division. It is noticeable here, however, that for the nucellus and pericarp at this stage, *Ivr2* expression is most closely associated with basal tissues. In many other instances, soluble invertase expression can be strongly associated with phloem unloading and rapid growth (Avigad 1982; Bret-Harte and Silk 1994). In other maize tissues, for example, relative *Ivr2* mRNA abundance and soluble invertase enzyme activity predominate in the most rapidly elongating, importing tissues and organs (Xu et al. 1996).

Shifts in localization of *Ivr2* mRNAs were also evident after the first 3 d of kernel development, when, in addition to label in the pericarp, *Ivr2* mRNA became evident in specific cells of the embryo/endosperm complex (Figure 3-6). At this stage of development, endosperm joins the pericarp and nucellus as a rapidly expanding tissue and eventually dominates kernel growth. Any role of soluble invertases in this starch-storing tissue appears to be very early and highly localized. (Insoluble invertases are later expressed in association with the endosperm/pedicel interface).

Little or no signal is detectable for *Ivr2* mRNA in kernels at developmental stages later than 7 days after pollination (Figure 3-7). This is consistent with northern-blot analysis of kernels from the same ears (Figure 3-2A). Results suggest that enlargement of the embryo and endosperm within the kernel has little or no association with activity of *Ivr2* soluble acid invertase after initial kernel expansion and seed set. Instead, the importance of *Ivr2* and soluble invertase activity appears to lie in early ovule development.

Discussion

Maize is central to U.S. agriculture with production more than double that of any other grain crop. It also provides an excellent model system for the study of physiology and molecular biology of vascular plants and grains in particular. Most previous research in kernel development has focused on the stages in which starch and protein storage take place (Thevenot et al. 1992; Giroux and Hannah 1994; Westgate 1994; Jones et al. 1996; Gao et al. 1998; Rastogi et al. 1998). Although some investigations have examined expression of specific genes during early kernel development (Somers et al. 1993, Giroux et al. 1994; Xu et al. 1996; Wittich and Vreugdenhil 1998; Scanlon and Myers 1998), results have typically been limited to the organ level. In maize pollination, double fertilization, and the initiation of embryo/endosperm development all occur in a very short period during the ovule/kernel transition. However, the large and spatially separated organs provide an intriguing opportunity to define, at the cellular level, how

these developmental signals affect expression of specific genes or vice versa. *In situ* hybridization techniques provide a valuable means of addressing this question.

Changes in invertase expression occurred during a key period in early kernel development (Figure 3-1). Abundance of mRNA for *Ivr2* soluble invertase increased markedly from ovules 2 days before silking, to kernels 4 days after pollination. Evidence suggests this period may be critical for defining kernel numbers and altering the extent of kernel abortion (Otegui and Bonhomme 1998). In addition, the *Ivr2* soluble invertase is closely linked to QTLs (quantitative trait loci) for carbohydrate metabolism, grain yield, and leaf expansion under drought stress (Pelleschi et al. 1999; Proul et al. 1999). Water shortage can strongly induce the activity of the vacuolar invertase in maize leaves, whereas the activity of cell wall invertase remains unchanged under these conditions (Proul et al. 1999). Presumably adjustments in osmotic pressure and water retention can be facilitated through conversion of sucrose to hexoses via soluble acid invertase. It is also notable that drought has an especially unfavorable effect on kernel set during the earliest phase of development (Schussler and Westgate 1991a, 1991b; Westgate 1994). ABA is considered to have a significant, negative role in this process (Cheikh and Jones 1994; Pack et al. 1997; Mambelli and Setter 1998). The response by vacuolar invertase to ABA provides a means by which kernel abortion could be partially countered under water shortage, possibly by aiding adjustment of osmotic pressure and concurrent sugar signaling inside the cells (especially in presence of cytokinins).

Immediately following pollination, soluble invertases are markedly upregulated relative to cell wall invertases and sucrose synthases. The vacuolar invertases are thus the first genes and enzymes of sucrose metabolism to rise in young developing kernels of

maize and also predominate among the primary enzymes for sucrose cleavage during this time (Xu et al 1996) In this instance, soluble invertase is not only central to provision of carbon skeletons and energy supply but also in generating sugar signals that can modulate sugar-responsive genes (Koch 1996 Wu and Koch unpublished data) Metabolism changes rapidly during pollination and cell division is initiated within hours of fertilization (Weier et al 1974 Kiesselbach 1980) Because many metabolic genes are responsive to sugar signals they are likely to be directly or indirectly affected by the activity of soluble invertase at this stage

Vacuolar invertase expression in young kernels appears to be markedly responsive to developmental signals from pollination and/or fertilization (Figure 3 2 and Figure 3-3) Pollinated kernels showed a stronger but shorter duration of elevated *Ivr2* mRNA levels and invertase activity compared to an extended period of limited expression in unpollinated ovules, regardless of whether samples were obtained from within the same ear or from different ears silking at the same time (Figure 3-3) The rapid change in soluble acid invertase expression associated with pollination and/or fertilization could be important to the first few days of kernel development This narrow window of soluble invertase expression shows a fascinating degree of similarity to the period critical for kernel set (Otegui and Bonhomme 1998) A possible physiological function for vacuolar invertase in early kernel development and set has been supported by this temporal association, and also by studies of changes to soluble invertase activity during stress-induced abortion (Pelleschi et al. 1999) In unpollinated ovules, the abundance of soluble invertase mRNA remains constant and drops gradually over a more extended period than observed in kernels A possible explanation for this is that

comparably less vacuolar invertase activity may be required for growth and/or sugar signaling prior to pollination/fertilization than after this event.

The abundance of soluble acid invertase mRNA also increased during the final two days before silking (Figures 3-1 and 3-2). These messenger RNAs were primarily localized in tissues around vascular bundles and the egg sac (Figure 3-4), consistent with the suggestion that these two regions are likely the primary sugar importing sites in ovules immediately before silking (Mogensen et al. 1995). Soluble invertase expression is altered by developmental signals (Figure 3-2) and shows differential cellular responses during ovule and early kernel growth (Figures 3-4, 3-5, and 3-6). In young kernels 2 days after pollination, vacuolar invertase mRNAs were specifically localized in tissues of the pericarp and vascular bundles (Figure 3-5). Pericarp is the fastest growing tissue at this stage (based on structure and volume analyses in Kiesselbach 1949) and strong expression of soluble invertase could provide hexose substrates for both growth and sugar signals (Koch 1996). At 4 days after pollination, the combined embryo/endosperm structure is readily visible in *in situ* analyses. At this point, basal cells in these new embryonic (and endosperm) tissues emerge sites of soluble invertase mRNA expression (Figure 3-6), consistent with their active metabolism and growth.

Increasing evidence has indicated that both sugar availability and water potential play critical roles in kernel set and final yield. Impaired photosynthate supply can strongly reduce the sugar content in kernels and decrease the percentage of kernel set (Wang et al. 1996). In addition, low water potential imposed on young kernels typically results in severe kernel abortion, but this effect can be partially reversed by feeding sucrose to ovaries via the stem (Zinselmeier et al. 1999). Ineffective utilization of sugar

is another potential contributor to kernel abortion (Schussler and Westgate 1995). High activities of both soluble and insoluble invertases early and later in kernel development, respectively, can theoretically facilitate sugar import and promote carbon metabolism. Finally, hexoses generated from sucrose cleavage by invertase can function as substrates for hexose-based sugar signaling, thus enhancing overall cell metabolism. Soluble invertase may thus be playing an important role in determination of kernel set and eventual yield.

Like many other plant tissues, maize kernel development is also tightly regulated by phytohormones. Cell division in kernels is strongly inhibited by water deficit (Artlip et al. 1995) and ABA accumulation (Manbelli and Setter 1998). Kernel abortion due to heat stress or ABA can be effectively reversed by elevated cytokinin levels (Cheikh and Jones 1994), suggesting that shifts in hormone balance may contribute to impaired kernel development in response to heat stress. Cytokinin has been implicated in N-based increases in grain yield (Smietiklas and Below 1992). Because vacuolar invertase is upregulated by cytokinin (Zeng et al. unpublished data), the possibility arises that soluble invertase may be involved in instances of cytokinin-improved productivity. It is intriguing that cytokinin levels in kernels rise rapidly after pollination (Dietrich et al. 1995; Banowetz et al. 1999), at least partly overlapping with increased soluble invertase activity (Figures 3-1, 3-2, and 3-3).

In kernels 7 to 10 days after pollination, messenger RNAs for vacuolar invertase were only immunonally detected in the embryo region (Figure 3-7). This result, plus previous analysis of total mRNA abundance (Figures 3-1 and 3-2), indicates that soluble invertase is no longer the primary enzyme for the first step of sucrose utilization at this

stage Instead cell wall invertase catalyzes sucrose cleavage during the later stages of kernel filling (Miller and Chourey 1992, Cheng and Chourey 1999) The shift of invertase expression from vacuolar isoforms to cell wall isoforms is consistent with the suggestion that the paths of import for entering sucrose change from primarily symplastic delivery in the enlarging maternal tissues of young kernels to the apoplastic transfer involved in growth of the embryo and endosperm

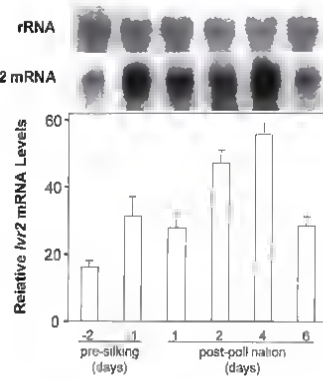


Figure 3 1 Relative abundance of *Ivr2* mRNA in maize ovules and kernels before silking and after pollination Maize hybrid plants (NK508) were grown under field conditions typical in north Florida (Spring crop) Samples of ovules and kernels were harvested at indicated intervals before silking and after pollination, respectively For each lane, 10 μg of total RNA were loaded and relative *Ivr2* mRNA levels were expressed relative to this standard. Error bars denote SEMs of three defferent experiments. In each instance separate ears were used for individual time points at which from 20 to 200 kernels or ovules harvested and processed as a pooled sample

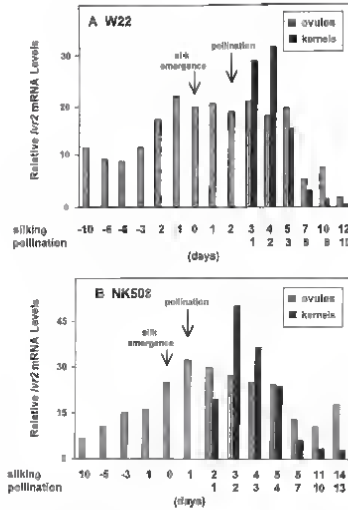


Figure 3-2 Relative abundance of *Ivr2* mRNA in maize ovules and kernels before silking and after pollination. Maize inbred (W22) and hybrid (NK508) plants were grown under field conditions typical in north Florida (Spring crop). Samples of ovules and kernels were harvested at indicated intervals before silking and after pollination, respectively. For each lane, 10 μg of total RNA were loaded and relative *Ivr2* mRNA levels were expressed relative to this standard.

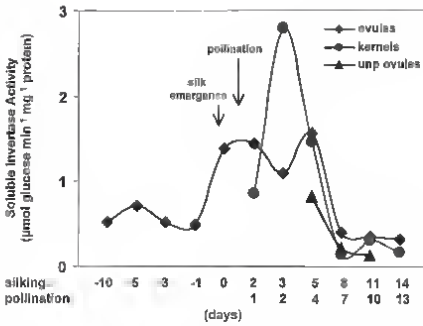


Figure 3-3 Soluble invertase activity in maize ovules (◆) kernels (●) and unpollinated ovules (▲) before silking and after pollination Maize hybrid plants (NK508) were grown under field conditions typical in north Florida (spring crop) Samples of ovules kernels and unpollinated ovules were harvested at indicated intervals before silking and after pollination respectively Ovules ovules from ears that were not pollinated at any point Kernels harvested after pollination Unpollinated ovules ovules harvested from partially pollinated ears on which developing kernels and unpollinated ovules could be clearly distinguished

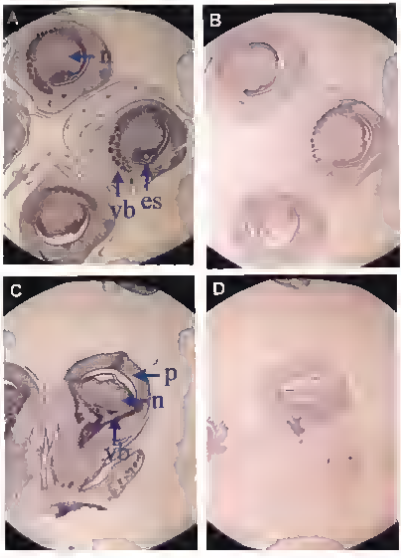


Figure 3-4 Localized expression of *Ivr2* soluble invertase in maize ovules 1 day before silking. Cross sections (A, B) and longitudinal sections (C, D) sections were probed with digoxigenin-UTP labeled antisense (A, C) and sense RNA (B, D). vb, vascular bundles, es, egg sac, n, nucellus, p, pericarp

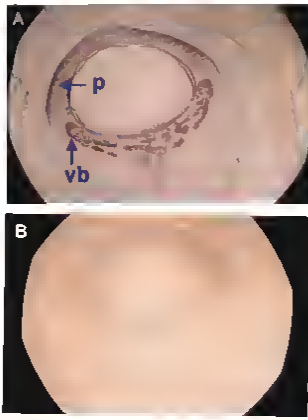


Figure 3-5 Localized expression of *Ivr2* soluble invertase mRNA in maize kernels 2 days after pollination Sections were probed with digoxigenin-UTP labeled antisense (A) and sense RNA (B) Note *Ivr2* labeling in vascular bundles (vb) and inner and outer cell layers of the pericarp (p)

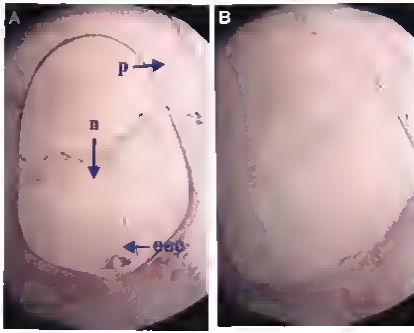


Figure 3-6 Localized expression of *Ivr2* soluble invertase in maize kernels 4 days after pollination Sections were probed with digoxigenin UTP labeled antisense (A) and sense RNA (B) Note labeling of *Ivr2* invertase mRNA in the pericarp and in specific cells of the embryo-endosperm complex. eee embryo-endosperm complex, n nucellus, p pericarp

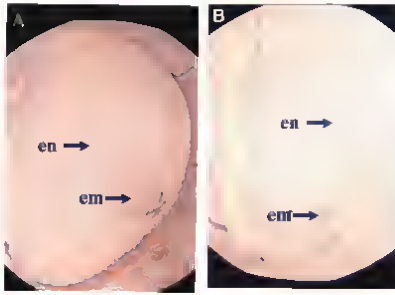


Figure 3-7 *In situ* analysis of *Ivr2* soluble invertase mRNA in maize kernels at 7 (A) and 10 (B) days after pollination. Sections were probed with digoxigenin-UTP labeled antisense and sense RNA. Note minimal label is evident for *Ivr2* mRNA at these stages of development. em. embryo, en. endosperm

CHAPTER 4
SITES OF SOLUBLE INVERTASE EXPRESSION, CELL EXPANSION AND EXTENT OF ROOT TIP ELONGATION ARE ALTERED WITH CYTOKININ AND SUGAR AVAILABILITY

Introduction

The maize root system consists of two parts: 1) primary or seminal roots, and 2) secondary or adventitious roots. These are sometimes called temporary and permanent roots, respectively, based on their presence throughout the life of the plants (Weier et al. 1974). Among all the tissues of the plant underground, root tips are the most active area of cell division, elongation, and differentiation (Kiesselbach 1949). They also function as architects of the entire root system. In addition, root tips provide an excellent model system for investigation of sugar responsive gene expression (Koch et al. 1992; Xu et al. 1996; Yu 1999 and the references there in), as well as metabolism of amino acids (Bronquiste et al. 1992) and fatty acids (Dreunde et al. 1992). Root tips have also been used to study phytohormone-mediated physiological and/or molecular responses (Sarquis et al. 1991; Bertell and Eliasson 1992; Sharp et al. 1994; Simonneau et al. 1998). In addition, growth of the entire plant can be modified by nutrient and/or phytohormone signals from the root system (Aiken and Smucker 1996 and references therein).

As carbon-importing structures, root tips need a constant supply of sucrose from phloem. Invertase or sucrose synthase initializes the first step of sucrose metabolism by

producing hexoses and/or UDP-glucose. Due to its vacuolar localization, soluble invertase is also important to turgor maintainence in growing root tips. In addition, invertase has the potential to exert a stronger influence on sugar-modulated gene expression via hexokinase-based signaling systems than does sucrose synthase (Koch 1996, Jang et al. 1997, Sheen et al. 1999, Koch et al. 2000) because invertase can provide two-fold more hexoses per sucrose cleaved.

Carbon metabolism can change markedly in excised maize root tips in response to sugar depletion (Brouquisse et al. 1991, Dieuaide-Noubhani et al. 1997). If exogenous sugars are not provided, sugar content and respiration rate decrease sharply after excision accompanied by reduced activity of enzymes for sugar metabolism and the TCA cycle (Brouquisse et al. 1991). Studies with ^{13}C labeled glucose revealed that flux through phosphoenolpyruvate carboxylase essentially ceases under sugar starvation. At the same time, contributions from acetyl-coenzyme A to glycolysis decrease gradually, and C from catabolism of protein and/or fatty acids rises (Brouquisse et al. 1992, Dieuaide et al. 1992, James et al. 1993, Dieuaide-Noubhani et al. 1997). During the initial 48 h of sugar depletion, protein degradation is correlated with a transient buildup of free amino acids. Activities of endopeptidases and carboxypeptidases increase throughout this period, and continue for an additional 48 h (James et al. 1993). After 96 h without an exogenous sugar supply, protein content of maize root tips decrease to 40% of its initial level (8% remained at 192 h) (Brouquisse et al. 1992). The endoproteolytic activities can be repressed by supplying external glucose at any time during this starvation period (James et al. 1993). Following 24 h of glucose starvation, the rate of fatty-acid oxidation increases two- to five-fold in maize root tips (Dieuaide et al. 1992). The results suggest

that proteins and lipids replace carbohydrates as the primary respiratory substrates when the sugar supply is limited

Not only metabolism is regulated by sugar availability, but also the extent and sites of expression for sugar modulated genes (Koch et al 1992, Wittich and Vreugdenhil 1998, Koch et al 2000) Although total enzyme activity of sucrose synthase shows little difference at either high or low sugar levels, changes occur in cellular localization of different sucrose synthase isozymes (Koch et al 1992) When glucose is abundant (2% [ca. 100 mM]) so too is the mRNA for *Sus1* sucrose synthase The SUS1 protein is also plentiful and is distributed mainly in the stele and apex of root tips When carbohydrate supply is limited (0 2% glucose [ca 10 mM]), the expression of *Sh1* sucrose synthase increases and SH1 protein is evident primarily in the epidermis (Koch et al 1992) The altered response of the two sucrose synthase genes to sugar availability at both molecular and cellular levels provides a potential mechanism for adjusting the capacity of sugar signaling and carbohydrate metabolism in importing cells relative to the levels of photosynthetic products

Symplastic movement appears to predominate as the primary means of sucrose entry into the apical meristem in roots (Giaquinta et al 1983, Bret-Harte and Silk 1994) Sucrose imported via plasmodesmatal pathways can be metabolized by either sucrose synthase or vacuolar invertase Because invertase can generate twice the "sensible" substrates as does sucrose synthase, it has the potential to more strongly influence sugar-modulated gene expression via hexokinase-based sugar signaling pathways (Koch 1996 Zeng et al 1999) Localized sites of invertase expression thus have the potential to both

directly and indirectly enhance growth of root tips since upregulation in specific cells has the capacity to amplify sugar signals as well as sucrose metabolism per se

Like other plant tissues, the growth of root tips is tightly regulated by phytohormones Tissues with elevated meristematic activity such as root tips have been characterized as having greater ratios of cytokinins/IAA than those of other tissues (Polevio and Polevio 1992) In primary root tips of maize, concentrations of IAA and ABA are greater in the growing tip than in more proximal regions (Polevio and Polevio 1992) Exogenous cytokinins inhibit root elongation, formation of lateral roots and induce swelling of root tips in etiolated pea seedlings (Bertell and Eliasson 1992) This response is correlated with moderate induction of ethylene and IAA synthesis within several hours of treatment. Ethylene also induces the swelling of root tips and mediates the increases in root diameter in response to mechanical impedance in maize (Sarons et al 1991) However the amount of ethylene produced in cytokinin treated root tips is generally considered too low to induce the swelling response (Bertell and Eliasson 1992)

High activity of invertase and sucrose synthase is often associated with rapid growth (Morris and Arthur 1984 Sung et al 1988). Deficiency in either of these two enzymes can potentially impair the development of sugar importing tissues Interestingly, invertase, but not sucrose synthase can be upregulated by cytokinins, suggesting 1) cytokinins may have a positive effect on invertase based amplification of sugar signals, 2) invertase may play a role in some aspect of cytokinin-regulated development, and/or 3) cytokinin effects on cell expansion may be at least partially mediated by soluble invertase Evidence also suggests that cytokinin enhancement of invertase may be involved in the events of initial reproductive development and meristem

formation (Siddeswar et al 1991 Ehness and Roitsch 1997, Kefi et al 2000) Resolution of invertase responses to cytokinins and sugars at a cellular level may provide important clues for defining the physiological significance of the changes observed The purpose of the following work is thus, first, to test the extent to which patterns of soluble invertase expression at a cell level are altered by changes in cytokinin and sugar availability and second, to determine the degree to which soluble invertase expression is associated with root tip elongation

Materials and Methods

Plant Material

Experimental material was obtained from the maize (*Zea mays L.*) hybrid NK508 Seeds treated with surface fungicide were germinated in the dark at 18°C on two layers of moist 3 MM paper (Whatman) in 27 x 39-cm glass pans Each pan was sealed with plastic and supplied with a continuous air flow of 1 L min^{-1} throughout the 4- to 5-d germination period. The terminal 1 cm or 0.5 cm were excised from the tips of primary roots and were immediately frozen in liquid N_2 or fixed in cold FAA (10% formalin, 5% acetic acid, 45% ethanol), respectively

For experimental treatments with external perturbation of sugar and plant growth regulators, root tips were cultured in modified MS medium using side-arm flasks with continuous air flow (1 L min^{-1}). Root tip lengths were measured carefully before and after 24-h incubations, and tissues were immediately frozen in liquid N_2

Frozen samples were ground into fine powder in liquid N_2. Total RNA was extracted as per McCarty (1986) and was quantified spectrophotometrically (Sambrook et al. 1989). Ten micrograms of total RNA were separated by electrophoresis in 1% agarose gels containing formaldehyde, transferred to a nylon membrane and fixed by 8 min of UV radiation. Resulting RNA blots were probed by hybridizing at 65°C with a maize *lvr2* cDNA (Xu et al. 1996) labeled by random primers in a solution with 7% (w/v) SDS, 250 mM Na_2HPO_4 (pH 7.2) and 1% BSA (Church and Gilbert 1984). Blots were washed and exposed against X-ray film with an intensifying screen at -80°C. The relative abundance of mRNA was quantified using a phosphor imager (Molecular Dynamics, Sunnyvale, CA).

Probes for *in situ* Hybridization

A fragment of the *lvr2* cDNA from 64 bp (NcoI) to 640 bp (NotI) was identified as having the least homology with its *lvr1* homologue. This fragment was subcloned into the NotI and HincII sites of a Bluescript II SK plasmid, which has T3 and T7 promoters at each side of its polylinker. DNA templates were linearized with either NotI or EcoRI for *in vitro* transcription, and driven by T3 or T7 promoters, respectively. Sense and antisense RNA probes were synthesized with digoxigenin-labeled UTP (Boehringer Mannheim) according to manufacturer's instructions. Probes were hydrolyzed with carbonate buffer (pH 10.2) at 60°C for 22 min to yield an average size of ca. 200 bp. For each slide, 25 ng of RNA probe was applied for *in situ* hybridization.

Tissue Preparation and *in situ* Hybridization

Samples were immediately fixed in FAA (10% formalin, 5% acetic acid, 45% ethanol) at 4°C overnight. Vacuum was applied during the first 2 hours of fixation. Fixed plant tissues were dehydrated with a series of increasing ethanol and Histo-Clear (National Diagnostics Atlanta, GA) levels before being embedded into paraplast. Embedded tissues were sectioned at 10 µm with a microtome and mounted on ProbeOn Plus microscope slides (Fisher Scientific). *In situ* hybridization procedures were conducted as per Jackson et al. (1994). Tissue sections were pre-treated with 0.2 M HCl for 20 min, 1 µg/ml proteinase K for 30 min, 4% paraformaldehyde for 10 min, and 0.5% (v/v) acetic anhydride for 10 min before being probed with labeled sense or antisense RNAs.

In situ hybridization was performed at 55°C overnight followed by two hours of washing with 0.2 x SSC at 50°C. Hybridized sections were treated with 20 µg/ml RNase A at 37°C for 30 min followed by another hour of stringent washing with 0.2 x SSC at 50°C. For immunological detection of hybridized RNA probes, sections were incubated with diluted alkaline phosphatase-conjugated anti-dig antibody (1:1000) for 2 hours at room temperature after incubating with 1% blocking agent (Boehringer Mannheim) and 1% BSA for 45 min each. Alkaline phosphatase activity was tested by incubating the sections with 100 mM Tris HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ containing a 2% (v/v) mixture of NBT and BCIP (Boehringer Mannheim) for 1 to 3 days at room temperature. Sections were dehydrated with an increasing series of ethanol and Histo-

Clear (National Diagnostics, Atlanta, GA) concentrations before being mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Results

In Situ Localization of *Ivr2* Soluble Invertase mRNA in Maize Root Tips

Figure 4-1 shows that mRNAs for *Ivr2* soluble acid invertase were localized in four regions within maize root tips. These were: 1) the entire length of epidermis distal from the root cap; 2) newly emergent, elongating cells in the cortex adjacent to the epidermis; 3) cells of the inner stele distal from the cortical zone, and 4) vascular regions from the beginning of their own differentiation zone to considerably distal from the apex. Each of the four regions was similar in having a potentially elevated demand for sugars and/or undergoing active expansion. Results were consistent with previous analysis of activity, which showed that invertases (soluble + insoluble) were detectable in whole root sections no further than 3 mm from the apex (Duke et al., 1991). In addition, soluble activity predominated over insoluble by 9:1 (Duke et al., 1991).

Growth and *In Situ* Localization of *Ivr2* Soluble Acid Invertase mRNA Is Altered by Sugar and Cytokinins

The growth rate of excised maize root tips was markedly responsive to the availability of sugar in 24-h experiments (Figure 4-2). Exogenous glucose at 2% (ca. 100 mM) increased the growth of root tips ca. 2-fold above that of sugar-depleted tips

(Sugar levels drop rapidly 6-8 h after excision [Brouguisse et al. 1991]). Kinetin (2 μM) alone had no obvious effects on root tip growth, however it substantially reduced the extent to which glucose could enhance root tip elongation. Root tips exposed to glucose + kinetin elongated only 70% to 80% as much as did those with glucose alone (Figure 4-2). In addition, root tip diameter increased significantly in the region about 1 to 4 mm behind the apex (Figure 4-4A). These morphological responses (especially the thickening) were similar in some respects to those reported previously for root tip responses to mechanical resistance or ethylene (Sarquis et al. 1991; Bertell and Eliasson 1992).

Concurrent *in situ* localization of mRNAs for the *Ivr2* soluble acid invertase indicated that without sugar or kinetin, soluble invertase was mainly expressed in epidermal regions accompanied by less pronounced expression in the stellar zones (Figure 4-3B). These sites of *Ivr2* expression were similar to those of SH1 sucrose synthase protein in sugar-depleted maize root tips (Koch et al. 1992). In the presence of 2% glucose, however, *Ivr2* soluble invertase mRNA was expressed almost evenly throughout the entire proximal region of maize root tips except for the root cap (Figure 4-3C). This result is similar to that for immunolocalization of sucrose synthase SUS1 protein in excised root tips under the same conditions (Koch et al. 1992).

Kinetin enhanced the expression of *Ivr2* soluble invertase mRNA in regions of epidermal and vascular tissues when sugar was depleted, but not in zones associated with root-tip elongation such as cortex and inner stele (Kiesselbach 1949; Weier et al. 1974) (Figure 4-3D). Results were consistent with the previous observation that elongation of maize root tips was unaffected by cytokinins when sugar was depleted (Figure 4-2).

despite increased abundance of *Ivr2* soluble invertase mRNAs in excised root tips under the same conditions (Zeng et al unpublished data) The enhanced expression of soluble invertase at the mRNA level by cytokinins appears not to be directly reflected in root-tip elongation

When both sugar (2% glucose [ca. 100 mM]) and kinetin (2 μM) were available mRNAs of *Ivr2* soluble invertase were evident primarily within the terminal 1 mm of the root tip (Figure 4-3E) Little or no expression of *Ivr2* soluble invertase mRNA was detected further than 1 mm distal from the apex, in either the cortical or inner stele tissues largely responsible for root-tip growth (Weier et al 1974 Kiesselbach 1980) Kinetin had a consistently negative effect on root-tip elongation when sugar was abundant (2% glucose [ca. 100 mM]) (Figure 4-4A B) However, it stimulated the transverse enlargement of cells just behind the apical region of the root tip (Figure 4-4A) At 2% glucose abundance of soluble invertase mRNA decreased 20% to 30% more when kinetin was present (Figure 4-4C) whereas the enzymatic activity of total soluble invertases remained relatively constant under the same conditions (Figure 4-4D)

To determine whether or not the swelling of maize root tips cultured with glucose and kinetin was mediated by ethylene signals, effects of an ethylene inhibitor and precursor were tested under the above conditions When 24-h incubations with kinetin were conducted in the presence of the ethylene biosynthesis inhibitor, AVG (aminoethoxy vinylglycine, 10 mM) glucose enhaced elongation rates by ca 20% more than otherwise observed (Figure 4-4A, B) In contrast, root swelling rather than elongation resulted from addition of the ethylene precursor ACC (1-aminocyclopropane-1-carboxylic acid, 10 mM) to MS medium with 2% glucose and thus physically

mimicked the appearance of kinetin-induced roots (Figure 4-4A, B). Ethylene involvement is thus implicated for some, but not all, of the kinetin-induced changes in root tips (e.g., gross morphology but not invertase gene expression or enzyme activity). Also, levels of *Ivr2* mRNA and soluble invertase enzyme activity did not appear to correspond to the degree of root-tip swelling (Figure 4-4C, D).

Discussion

Sugar and phytohormones regulate many aspects of growth and development in vascular plants. Both sugar and phytohormone signaling systems are involved in communication between distant organs, where they provide valuable information on internal and external conditions at different developmental stages. Sugar can have phyto hormone like effects on plant development that extend beyond those of a simple C supply. Sugars up- or down-regulate many specific genes, including those involved in classic developmental and phytohormone responses (Koch 1996, Azuma et al. 1997, Kurata and Yamamoto 1998, Koch 2000). Recent results have also indicated extensive crosstalk between sugar and cytokinin signals (Moore-Gordon et al. 1998, Charriere et al. 1999, Zeng et al. unpublished data).

The present work presents data for *in situ* localization of *Ivr2* invertase mRNA, which indicates that sites of expression can change depending on the sugar or phytohormone signals involved. Such shifts are significant because they 1) have the potential to alter sugar signals at a cell level, and 2) can change the capacity for sucrose import and growth among individual cells. Results shown here also indicate that soluble

invertase expression may be linked to carbohydrate-based differences in cell expansion and root elongation but not necessarily when growth is perturbed by phytohormones

The four regions of soluble invertase expression evident in growing maize root tips (Figure 4 1) corresponded to sites likely to be especially active in sucrose import and/or utilization (Kiesselbach 1949 Weier et al 1974) The first of these, the single-celled epidermal layer gives rise to root hairs and is also a primary site of water and nutrient uptake in many grasses (Weier et al 1974 Varney et al 1993 McCully 1999) Associated metabolic demands of these cells are thus likely to be well above those of the adjacent cortex Both invertase and sucrose synthase up regulation could provide needed substrates To fact, invertase localization in this tissue is similar to that observed for the SH1 sucrose synthase which in addition to providing substrates was also suggested to confer import priority during stress (Koch et al 1992) This is consistent with a vital role for root epidermis under diverse conditions (McCully 1999)

Localization of soluble invertase mRNA for *Ivr2* in vascular bundles may be related to the high energy requirement estimated for phloem, and/or osmotic balance in nearby cells Phloem loading and unloading are often associated with ATP hydrolysis (Martin et al 1993 Lappartient and Touraine 1996 Moriau et al 1999) Companion cells adjacent to sieve element are considered the probable source of ATP for loading and unloading and sucrose synthase has been localized specifically in these cells (Nolte and Koch 1993) PmSUC2 protein, a sucrose carrier is also labeled specifically in companion cells (Stadler et al 1995) A possible role for soluble invertase in vascular tissues of root tips may thus be that of aiding symplastic unloading and possibly also the elongation of vascular cells during their early development.

Expression of the *Ivr2* soluble invertase in the cortex and inner stele may be related to both storage and turgor function in these cells. These tissues are composed mainly of storage parenchyma cells with large intercellular spaces (Weier et al 1974) Imported sucrose can be temporarily stored as sucrose hexoses, or starch when the supply from photosynthetic tissues is abundant (Winter et al 1974) Sucrose synthase reactions typically predominate during starch biosynthesis, but soluble invertases could be important during sugar storage In particular hydrolysis of sucrose into glucose and fructose inside vacuoles can increase turgor pressure in parenchema cells of the cortex and inner stele where this organelle typically occupies more than 90% of the total cell volume (Weier et al 1974) Turgor in cells of the elongation zone can push the root apex forward, overcoming soil resistance (Bengough et al 1997) Continuous elongation of maize root tips requires turgor pressure above a threshold of about 0 45 MPa (Frensch and Hsiao 1994) Elongation of roots in pine seedlings for example is limited under high osmotic stress due to decreased cell turgor (Triboulot et al 1995) Under conditions of water stress, maintenance of root elongation can result in plant access to otherwise unavailable moisture sources Such root tip elongation under low water potential requires ABA (Sharp et al 1994), which accumulates in root tips under drought stress (Zhang 1994 Zhang and Tardieu 1996, Simonneau et al 1998) Soluble invertase is also upregulated by ABA in root tips (Zeng et al unpublished data) and its activity is potentially important for symplastic phloem unloading and turgor maintenance in these tissues The symplastic route appears more important than the apoplastic pathway in root tips (Pritchard 1994 Bret-Harte and Silk 1994), and evidence suggests that the rate of this solute import into the growth region may be critical for cell elongation under water

stress (Fromsch and Hsiao 1994) Soluble invertase may thus have a significant role in root-tip elongation that extends beyond turgor alone.

Data in Figure 4-2 show that elongation of maize root tips is very sensitive to sugar availability Cell expansion in barley roots increases with sugar availability and is inhibited in the presence of a non metabolizeable sugar analog (Farrar et al 1995) Sugar appears to play a dual role in this instance. It serves not only as an essential carbohydrate source for metabolism, but also provides substrates for sugar signaling (Koch 1996 Sheen et al 1999) Plentiful supplies of photoassimilates typically up-regulate specific genes which in turn can promote growth and facilitate uptake of nutrients in root systems (Koch 1996 Koch 1997) More N can also be absorbed by a larger root system thus contributing to a more optimal balance of C/N and favoring protein synthesis (Koch 1997) Induction of the S28 ribosomal protein by sugar abundance in maize roots (Chevalier et al. 1996) indicates that the overall protein synthesis may also be increased under these conditions.

The expression of soluble invertase at a cellular level in excised maize root tips responds within 24 h to changes in glucose availability (Figure 4-3B C) Without an external sugar supply carbohydrate content decreases sharply after excision, and is accompanied by reduced activities of enzymes for sugar metabolism and the TCA cycle (Brouquisse et al 1991). Therefore, when excised root tips are cultured in sugar-free medium for 24 h, internal carbohydrates are nearly depleted and tissues are starvation stressed. In this instance, soluble invertase is expressed primarily in epidermal cells and to a lesser degree in the stelar zone (Figure 4 3B) A similar pattern was previously observed for the SH1 sucrose synthase under carbohydrate deprivation (Koch et al 1992)

Both observations are consistent with the suggestion that under starvation conditions, survival of root tips may depend on delivery of limited resources to the most vital cells and tissues.

In Figure 4-3C, the *Ivr2* soluble invertase mRNA is shown to be expressed at relatively similar levels throughout tissues proximal to the apical 2 mm of the root tips when sugar is abundant. This result is similar to the immunolocalization of SUS1 sucrose synthase protein in excised root tips under similar conditions (Koch et al. 1992). Both *Ivr2* soluble invertase (Xu et al. 1996) and *Sus1* sucrose synthase (Koch et al. 1992) are upregulated by elevated levels of carbohydrates. They are also associated with symplastic unloading of sucrose and generation of substrates for hexokinase-based sugar signaling when photoassimilate supply is abundant. The expression of soluble invertase in zones of the cortex and inner stele thus could facilitate a more global enhancement of carbohydrate import and turgor across many cell types and tissues during root elongation (Frensch and Hsiao 1994, Bengough et al. 1997).

The *in situ* localization in Figure 4-3D revealed enhanced levels of *Ivr2* soluble invertase mRNAs in regions of epidermal and vascular tissues when kinetin was present in sugar-depleted root tips. In contrast, there was minimal *Ivr2* mRNA in zones likely to contribute more prominently to root-tip elongation, such as the cortex and inner stele. This is consistent with an apparent lack of immediate association between cytokinin-enhanced expression of soluble invertases and root tip elongation in the absence of sugars. Although elongation of sugar-depleted maize root tips is unaffected by cytokinins (Figure 4-2), abundance of soluble invertase mRNAs is increased in excised root tips under the same conditions (Zeng et al. unpublished data).

Cell-level expression of *lvr2* soluble invertase also changed in response to kinetin when sugar supplies were abundant (2% glucose [ca. 100 mM]) (Figure 4-3E) Messenger RNAs for *lvr2* soluble invertase were evident primarily within the 1 mm terminal zone of the root tips where meristemic and newly-expanding cells predominate (Kiesselbach 1949, Weier et al 1974) Little or no *lvr2* soluble invertase mRNA was detected further than 1 mm distal from the apex in either the cortical or stellar zones largely responsible for root tip elongation (Kiesselbach 1949 Weier et al 1974) This result was consistent with the previous observation that kinetin had a negative effect on root tip elongation (Figure 4-2A,B)

The swelling of maize root tips in the presence of kinetin when sugars were abundant (Figure 4-4A) is visually similar to the root tip ethylene response (Sarquis et al 1991 Bertell and Eliasson 1992) Nevertheless, the extent of swelling induced by ACC (an ethylene precursor) is markedly less than that resulting from kinetin at the same sugar level (Figure 4-4A) This indicates that cytokinins may regulate the growth process of root tips via multiple mechanisms, possibly including at least one ethylene-responsive pathway

There are two possibilities for the more rapid root tip elongation in the presence of AVG (Figure 4-4A) First, since AVG blocks ethylene biosynthesis (Meravy et al 1991, Berglund and Oblsson 1992 Gob et al 1997, Child et al 1998) at least some portion of the kinetin-induced swelling and growth inhibition is probably mediated by ethylene In the presence of AVG and abundant sugars, root tips elongated rapidly and were not inhibited by cytokinin, suggesting ethylene is involved in cytokinin-inhibited root growth Second AVG effects on *lvr2* mRNA may have resulted from its broad

spectrum inhibition of pyridoxal P-based enzyme reactions. In either instance, a clear distinction is shown here between sugar based changes in root elongation that are associated with altered expression of *Ivr2* soluble invertase and other perturbations of root tip growth that are independent of this expression.

In conclusion, expression of soluble invertase in root tips is associated with carbohydrate-based differences in root-tip growth, but not necessarily other effects (eg kinetin- or ethylene-induced differences). Glucose promotes root-tip elongation and *Ivr2* soluble invertase expression, whereas this process is inhibited by cytokinins. However the relationship between soluble invertase expression and root tip elongation did not extend to changes induced by ethylene inhibitors (AVG) or precursors (ACC). In fact, the ethylene precursor ACC dramatically reduced expression of the *Ivr2* invertase and its contribution to root elongation (and/or cellular enlargement).

The above results support two suggestions. First, the expression of soluble invertase mRNAs in the elongation zone appears to be associated with carbohydrate-based root tip elongation. Threshold turgor pressure has proven to be essential for root-tip elongation (Frensch and Hsiao 1994; Triboulot et al. 1995; Bengough et al. 1997). Hexose products from soluble invertase activity are important to this turgor, and may dominate in some instances but not others. Secondly, soluble invertases are also expressed in regions where sucrose import and/or utilization are most pronounced even under adverse physiological conditions. These sites, the epidermis, vascular bundles, cortex, and apex, could thus be affected by the soluble invertase contributions to both C-demand and hexose-based sugar signaling.

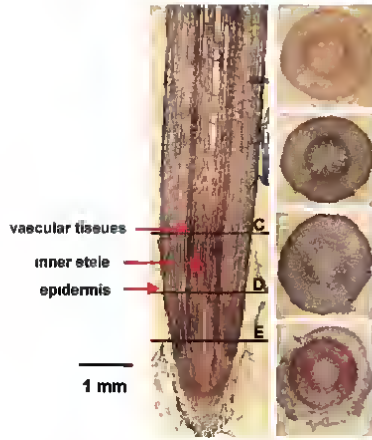


Figure 4-1 *In situ* localization of *Ivr2* soluble acid invertase mRNA in maize seedling root tips Sections were probed with digoxigenin-UTP labeled antisense (A, C, D, E) or sense control RNA (B)

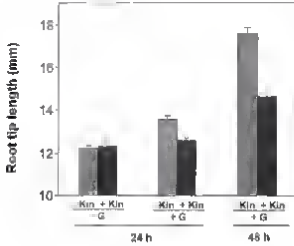


Figure 4-2. Elongation of maize seedling root tips with or without glucose (2% [ca. 100 mM]) and with or without kinetin (2 µM). Maize (hybrid NK508) seeds were germinated in the dark at 18°C for 4 to 5 d until the primary roots reached 4 – 5 cm. The terminal 10 mm were excised and cultured for 24 or 48 h in MS media supplemented as above. Error bars denote SEMs of three different experiments. For each experiment, 30 to 40 excised root tips were processed as a pooled sample.

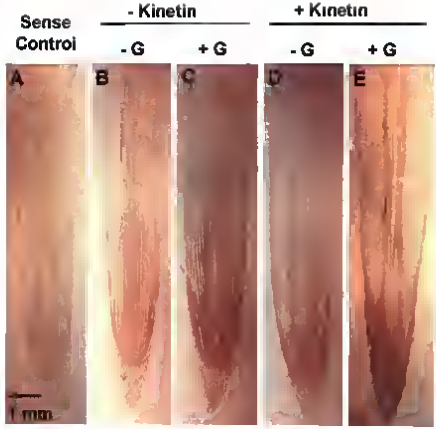


Figure 4-3 *In situ* localization of *Ivr2* soluble acid invertase mRNA in maize seedling root tips. Sections were probed with digoxigenin-UTP labeled antisense or sense control RNA. Maize (hybrid NK508) seeds were germinated in the dark at 18°C for 4 to 5 d until the primary roots reached 4 - 5 cm. The terminal 10 mm were excised and cultured for 24 h in MS media with or without glucose (2% [ca. 100 mM]) and with or without kinetin (2 μM).

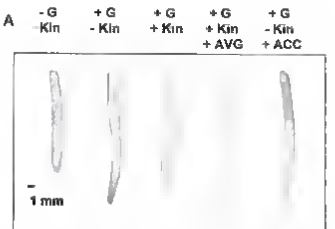
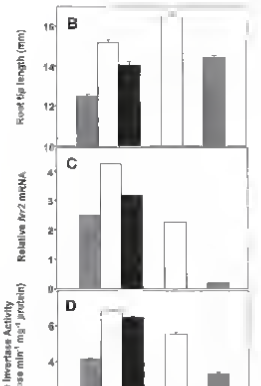


Figure 4-4 Effect of AVG and ACC on root elongation and *Ivr2* invertase expression with or without glucose and kinetin Maize (hybrid NK508) seeds were germinated in the dark at 18°C for 4 to 5 d until the primary roots reached 4 5 cm The terminal 10 mm were excised and cultured for 24 h in MS media with or without glucose (2% [ca. 100 mM]) and with or without kinetin (2 μM) In addition glucose + cytokinin responses were tested in the presence of the ethylene inhibitor AVG (10 mM), and glucose effects were examined with the ethylene precursor ACC (10 mM) Error bars denote SEMs of three different experiments For each experiment 30 to 40 excised root tips were processed as a pooled sample



CHAPTER 5
CLONING AND MOLECULAR ANALYSIS OF GENOMIC DNA FOR SOLUBLE MAIZE INVERTASES *IVR2A* AND *IVR2B*

Introduction

Invertase cleaves sucrose into fructose and glucose, thus catalyzing the first step in C metabolism by many sucrose importing plant organs. Due to the importance of this enzyme to allocation of resources within plants, it has been extensively studied for a number of decades (for example, Straus 1962; Ricardo 1974; Nakamura et al. 1988; Xu et al. 1996). Multiple isoforms of invertase have been characterized, based primarily on different subcellular locations and pH optima. Soluble invertases typically have pH optima ranging from neutral (pH 7.5) to acidic (pH 4.5), and have been identified in cytosol (Karuppiah et al. 1989; Fahrendorf and Beck 1990) and vacuoles (Leigh et al. 1979; Giaquinta et al. 1983), respectively. The latter may also be secreted as a soluble extra-cellular enzyme in some instances (Sturm 1999). Insoluble invertases typically have pH optima from 4.0 to 5.3 and show varying degrees of ionic bonding to cell walls (Straus 1962; Fahrendorf and Beck 1990).

Soluble forms of invertase are typically associated with rapid growth, such as occurs in young carrot seedlings (Ricardo and ap Rees 1970), during leaf enlargement (Schaffer et al. 1987), and in early growth of young fruit and seeds development (Sung et

of photosynthetic products can upregulate the *Ivr2A* soluble invertase, which in turn may enhance import of sucrose into cells and generate more substrates for sugar signaling.

A partial sequence of the *Ivr2A* cDNA has been characterized (Xu et al. 1995 Genbank Entrez, U31451). The NDPNG consensus sequence and its upstream region were not present in the initial clone. Unique features of the *Ivr2A* invertase expression in specific tissues at key stages of development (Xu et al. 1996, Chapter 3, and Chapter 4) suggested that important regulatory information may lie in these genes. For this reason its genomic sequence was sought, with particular attention to the promoter region and the leading introns/exons. This chapter presents the cloning and molecular analysis of genomic DNA for the maize *Ivr2A* and *Ivr2B* soluble invertases including upstream promoters and 5' coding regions.

Materials and Methods

Probes

The probe used to obtain *Ivr2* genomic clones was a 576-bp NcoI-NotI fragment of the *Ivr2A* cDNA (positioned between bp# 64 and bp# 640 of the initial cDNA sequence [Genbank Entrez, U31451] and 130 bp downstream of the NDPNG). It was subcloned into HincII and NotI sites of a Bluescript II SK plasmid. The recombinant plasmid was amplified in *E. coli* XL-1 cells and purified (Plasmid Kit, Qiagen, Valencia, CA). The inserted partial-*Ivr2A* fragment was excised from the vector, purified, and used

as a random primer template for synthesis of ^{32}P labeled probes. Probes were used to screen a genome library from maize (Clontech, Palo Alto, CA).

Screening

The maize genomic library was screened as described by Sambrook et al. (1989). Plaques or colonies were blotted to nylon membranes, and DNA was denatured *in situ* with 0.5 M NaOH, neutralized with 1.0 M Tris-HCl buffer (pH 7.5), and fixed by 8 min of UV radiation. Membranes were hybridized at 65°C in a solution with the *fw2A* cDNA probe, 7% SDS, 250 mM Na_2HPO_4 (pH 7.2), and 1% BSA (Koch et al. 1992). Blots were washed and exposed against X-ray film with an intensifying screen at -80°C. Two strongly hybridizing, independent clones were isolated, purified, and subcloned in Bluescript II SK plasmids.

Sequence Analysis

The subcloned genomic DNAs were sequenced at the University of Florida Sequence Core Lab. Results were compared to sequences for other invertases in the Genbank. Computer assisted analyses of DNA and protein sequences were conducted with Blocks Multiple Alignment Processor (Fred Hutchinson Cancer Research Center, Seattle, WA).

Results

Two clones from a maize genomic library hybridized strongly to the *Ivr2A* cDNA probe. These two were isolated, purified, subcloned, and sequenced. They were designated *Ivr2A* and *Ivr2B*. Both *Ivr2A* and *Ivr2B* have an extremely GC-rich domain near the 5' end of their coding region, about 75% and 78% GC respectively. *Ivr2A* has a 99% identity to the *Ivr2A* cDNA sequence published in genbank whereas that of *Ivr2B* is 92% similar.

Figure 5-1 shows the DNA sequence of the 5' leading exons, introns and promoter region of the *Ivr2A* invertase. A TATAA box was defined betwen -51 and -46 upstream of the translational start codon (ATG). Two ACGT cores were located, one positioned between nucleotides -432 and -449 and another between nucleotides -841 and -838. Structures similar to ABA response elements were also identified in the 5' promoter region from -589 to -580 (GCCGAGTGTC) and from -71 to -64 (AGGCACCGA) (Figure 5-1). These respectively shared key features with a motif III (GCCGCGTGGC) in the *Rab16* gene of rice (Ono et al. 1996) and a CE1 (TGCCACCGG) in the *HVA1* gene of barley (Shen and Ho 1995).

A distinct feature of the *Ivr2A* gene is that it has a very tiny first exon and very small first intron (Figure 5-2). *Ivr2A* also differs from most other invertases, including the *Ivr1A* soluble invertase of maize (Xu et al. 1995), in its lack of intron divisions within the NDPNG consensus region. Other invertases typically have two amino acids (PN) at the center of their consensus NDPNG sequence encoded by a small, isolated, 9 bp exon

(Figure 5-2). Instead, *Ivr2A* has its NDPNG encoded nearly intact, at the end of the second exon.

Ivr2B is also an unusual invertase. It, too, lacks the distinctive small exon positioning in the center of its consensus NDPNG sequence (Figure 5-2). Like *Ivr2A*, its NDPNG is encoded by a region at the end of a relatively large, upstream exon, exon #1 for *Ivr2B* and exon #2 for *Ivr2A*. In addition, the intron following the NDPNG of *Ivr2B* has only 82 bp, and is thus substantially smaller than that of other known invertase introns at this position.

A blast search revealed little or no similarity between the N terminal amino acid sequences of either *Ivr2A* or *Ivr2B* and that of other invertases (Figure 5-3). A high degree of similarity among all three maize soluble invertases was evident downstream of position 160 in Figure 5-3. Strong hydrophobic regions were also identified between positions 78 and 100 among these invertases. Although the amino acid sequences of these regions share little similarity between *Ivr1A*, *Ivr2A*, and *Ivr2B*, SignalP Prediction (Nielsen et al. 1997) analysis indicated that these regions could serve as signal peptides for these invertases. The putative cleavage site for *Ivr1A* is at position 99 whereas that for *Ivr2A* and *Ivr2B* is at position 111 (Figure 5-3).

A marked degree of GC-richness was evident in the 5' region of all three cDNAs (*Ivr1*, *Ivr2A*, and *Ivr2B*), which resulted in Tm values that typically rose well above 94°C (Figure 5-4). Since this is the temperature typically used for opening double-stranded DNA chains during standard PCR-based procedures, the GC-rich soluble invertases may be under-represented in amplified libraries and/or resistant to analysis by PCR-based procedures. Tm values for *Ivr2A* and *Ivr2B* are 5 to 10 degrees higher than for *Ivr1A*

The derived sequences of the three maize soluble invertase proteins were compared with other soluble and cell wall invertases from various species A phylogenetic tree was generated (Figure 5 5) based on the Block Multiple Alignment Processor (Fred Hutchinson Cancer Research Center, Seattle, WA) Results indicate that evolutionary divergence between soluble and cell wall invertases occurred early Maize soluble and insoluble invertases divided into groups along lines evident in many diverse species In addition, the *Ivr1* and *Ivr2* maize invertases appear to have diverged from one another prior to the separation between the maize *Ivr1A* and the soluble invertases of rice. Not surprisingly the divergence of maize *Ivr2A* and *Ivr2B* soluble invertases appears to have taken place most recently

Discussion

The sugar and ABA responsiveness of the soluble invertase genes in maize may involve to the ACGT cores identified in the upstream promoter regions of both *Ivr2A* and *Ivr2B* (Figure 5-1 Figure 5-2) This sequence of four-base pairs has been widely observed among cores of carbohydrate and ABA responsive elements, although flanking sequences are typically divergent among species and between elements (Table 5-1). The ACGT sequences, together with ACT, CE1 and MotifIII like elements, in the *Ivr2A* and *Ivr2B* promoter regions may thus contribute to the sugar and/or ABA-regulated responses of these genes observed at the mRNA and enzyme activity levels (Xu et al 1996. Zeng et al unpublished data)

Sugar responsiveness of the *Ivr2* soluble invertases may also involve a separate set of possible ACT cores two of which were identified in the *Ivr2A* promoter region between positions -468 and -466, and between -567 and -565 (Figure 5.1). ACT cores are a common feature among sugar regulated cis-elements (Ishiguro and Nakamura 1992 Grierson et al. 1994, Kaytor et al. 1997). However, flanking sequences of the putative ACT cores in the *Ivr2A* promoter do not share similarity with other more common flanking regions.

A distinguishing feature of the *Ivr2A* and *Ivr2B* genes is that both lack intron/exon divisions within the consensus NDPNG (Figure 5.2). These divisions otherwise result in the more typical formation of a very small 9 bp exon (exon 2) at the center of the DPNG sequence (Xu et al., 1995). The pentapeptide NDPNG is also called the βF motif, which is located near the N terminus of mature invertase proteins and is assumed to be important for catalytic activity (Sturm 1999). To date, almost all reported genomic sequences for plant invertases contain the small, 9 bp second exon encoding a core dipeptide of the NDPNG (Table 5.2). Other than the soluble invertases of maize, a carrot gene for cell wall invertase is one of few known exceptions.

The functional significance of this unusual intron-exon arrangement remains unclear. Exon skipping has been reported during alternative splicing in potato invertases (Bournay et al. 1996). Although no aberrant invertase transcripts were observed under non-stressed conditions, cold stress apparently altered RNA processing such that the tiny exon was deleted from a small percentage of the invertase transcripts (Bournay et al. 1996). Whether or not this production of altered transcripts could be advantageous in any way remains unclear. Disruption of the NDPNG would presumably leave the resultant

protein disfunctional as an invertase, but alternate roles are possible (enzymatic or other). Invertases lacking this configuration, such as *Ivr2A* and *Ivr2B*, may have advantages under specific conditions, especially if rapid induction or expression under stress are involved. Because the 9-bp exon is so small, it is likely to require complex transcriptional events for precise splicing (Mary Schuller, personal communication). This may pose minimal difficulty under most environmental conditions, but under stress key processing factors (or events) may become limiting.

A hydrophobic region is a common feature in the N terminal regions of the amino acid sequences for soluble invertases, although sequence similarity is minimal (Figure 5-3). PSORT analysis predicted these signal peptides (Nielsen et al. 1997) to target the soluble invertase proteins to the ER, where most vacuolar proteins are initially modified enroute to their final sites (PSORT www Server: http://psort.nibb.ac.jp/).

The 5' GC-richness of *Ivr2A* and *Ivr2B* invertase genes apparently contributed to initial difficulties obtaining full length *Ivr2* cDNAs: since resultant Tm values indicated these regions would not likely respond to standard PCR for either library amplification or RT-PCR (Figure 5-4). The average GC level in the first two exons of *Ivr2A* and first exon of *Ivr2B* was above 80%. In some regions, GC levels were as high as 90%. In the other coding regions of *Ivr2A* and *Ivr2B* invertases, the GC content was around 60 to 70%. The high GC percentage in the leading exons resulted in a Tm value well above 94°C, the temperature used for opening double DNA chains during standard PCR based procedures.

Analysis of phylogenetic relationships among the three maize soluble invertase genes and a comparison to other invertases clearly indicates that the divergence of

soluble and cell wall invertases took place much earlier than sub-divisions among either group (Figure 5-5). The three soluble invertases of maize examined thus far, *Ivr1A* (Xu et al. 1995), *Ivr2A* and *Ivr2B* show a high degree of sequence similarity to other soluble invertases, such as those from rice (Lin et al. 1997), citrus (Chen et al. 1997), and tomato (Ohyama et al. 1998). The cell wall invertases of maize, *Incw1*, *Incw2*, and *Incw4* (Shanker 1995; Kim et al. 1999; Taliercio et al. 1999) also share sequence similarity with other invertases in the cell wall class from goosefoot (Rotsch et al. 1995) and pea (Buchner 1997). The amino acid sequences of *Ivr2A* and *Ivr2B* have the most extensive homology, whereas that of the maize *Ivr1A* invertase indicates a closer relationship to a different soluble invertase in rice. Interestingly, the sequences of maize cell wall invertase *incw3* (Taliercio et al. 1999), and pea cell wall invertase *cw1* (Zhang et al. 1996) share more sequence similarity to soluble invertases than to other cell wall invertases. Evidence has indicated that some vacuolar forms of invertase can be sorted to extracellular space without first moving into vacuoles (reviewed by Sturm, 1999). Either way, phylogenetic analyses in Figure 5-5 group the maize *Incw3* and pea *cw1* invertases into a soluble-invertase category based on other aspects of sequence similarity.

In summary, molecular analyses of three genes for soluble invertases in maize reveal 1) the presence of several cores for putative sugar- and ABA-reponse elements, 2) an unusual intron/exon arrangement indicating that the *Ivr2* invertases do not encode their NDPNG consensus sequence using the very small exon typical of other invertases, 3) an unusually high GC content in the 5' coding sequence of these genes (Tm > 95°C), and 4) phylogenetic analyses that indicate early divergence not only of soluble and insoluble invertases, but also between *Ivr1* and *Ivr2* subgroups of soluble invertases.

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AGGACCATATGAGCCGGTGAGGGAGAGGATGGAGGCGTCGCTTTCCAAAT
CGCCGAAGAGTCGTCAGGAGCGGAGGAGCCGAGGAGTCGCGGACGTTCG
AGMAGAGTGAGTGAGTTGTGAGAGTGAGAGAGAGGTGACGTGAGGGAC    (ACGT core)
GGGGGAGGGGGTATGTGTTATTACGCTCGTCGCTAACCGTGCCTAAACCC
TAATCGTGTCGTGCCTGTGCCGGCCACCGTACCCACACTCGGCCCAAG
CACGGCACTAGAGGTCGGGCGTGCCGGCACGGGCCCGCCTCTAGTCGTG
TCGTGCCGTGCTTGGGCACGGTGGGCCGTGTCGTGCCTCGTGCCGGCCG
GTTAAGGCGGCACTACTGGCCATCTATAGCAAAGTCTTTGCCGAGTGTCC    (MotifIII like element)
GAAAAAGTACTCGGCAAAATCTTTTGCCGATAAAATATTTGTTTGAGTGTT    (ACT core)
ACACTCGGCAAAGCTTTTGTCGAGTGTAAAATACATTTGCCGAGTATCG
GCTAAGAACGCGATTTCGGTAAAGCAACTACGTTCCATTTTATCAATATA    (ACGT core)
AAAGAAAAAATAATAATAATAATCCAACCAACAGCAGCAGCAACAACAAA
GTTCATAACGATATGTTCAGCAGGGTCCTTGAGATTCGACAGTTGCCATT
ATATGGAGACGGTGATCGGGGATCGAAGAACACGGGCGGGGCCCAGGAAA
CACATGACAGAGTGAAGAAATAATTGGTCCAATGGGGGGGGTGCCATGTGCT
GGACAGATGGGCTGGCAGGGCAGATCGGATCCGGATGTGAAAAATTCAACA
TCAGCTCCGTACCNGACCNGACCAGACCAGTGACGAGAGCCGAGACCGGC
CTTGGAAAAAGGCGAGGAAGGAGGCTTGGAGGCTGGAGGAGGAGGAGGA
AAGCGAGGACCGATCCCCAACCACACTATAAATACGATCCCGCAATGCC    (CE1 like element
CTCTCTCTCCCATCCCACCCCTCGCCCAATGCGTAGTTGTATCTGCATCT    and TATAA box)
CCAACACCACCAGCAGAGGTTCCAGTTAGTTTTTTTAGATTAGAAAAAT    (1st intron)
CHAGTCCAACAGACTCGCCGCACTGCGCGGGATCGACCTCGTCGCAACTC
CCGTGTGCGCCGCCAATAATGGAGACCCGGGACACGGATGCGACGCCGCT
CCCCTACTCGTACACGCCGCTGCCGGCCGCCGACGCCGCGTCGGCCGAGG
TCTCCGGCACCGCGCAGGACGCGGAGCACGCGGCGGCCCCTCTGCGCGCGG
GCGCTCGTGCTCTCCGCCGCGCTGCTCCTAGCCGTGGCCGCGCTCGTCGG
CGTCGGTAGCCGCCCGGCGCGTGGGGATGACAGATCGGGCGCCTCGT
CGCCGACGCCGAGCAGGAGCAGGGGCCCGAGCCCGGCGTGTCCGAGAAG
ACGTCCGGCGCGTCTGACGACGGCGGCAGGCTCCGTGGAGCTGGCGGGAA
CGCCTTCCCGTGGAGCAATGCGATGCTGCAGTGGCAGCGCACGGGATTCC
ACTTCCAGCCGCAGAAGAACTGGATGAACGACCCCAATGGTACGTTACGA    (NDPNG)
TCCTCTCTCTCTCTCTCTCTCTCTCTCTCTGCTGCTGCCCGCTGCTGCATT
TGGTTTGGGTTGCATCCCAACTCACATCCCCTTCCAAACGACTATGGAT
TAGATCGATTTGAACCACATATGAACGAATTAATTGTCCACGCATTTGATTC
CTTTCACGTGCATTTCATCCAACGAGGCGCTTGGCACCACACCCATGGAC
GAATATTCAGCGGGCCCCTTTTTTCTCTTCACTTTCCCTTTTTCTCT
TCACTTTTCCCTTTTTTTTTTCTTTCTCATCCACATACAAATTCCAGATA
CCCCCGGGCCCTCCGGGATTCCACCCAAACCACCAGGCCTACCGTTTGTGTA
TTCTTCTTTGTTTTTCTGCCCTTCTCGTGCCTGACTTGTTTGGTCCAAG
TCGTCGTCGCTTCGTAATAATAATAATAATTGTAAATTATACGCTTGCA
GCCCCCGTGTACTACAAGGGCTGGTACCACCTCTTCTACCAGTACAACCC
TGACGGCGCCATCTGGGGCAACAAGATCGCGTGGGGCCACGCCGTGTCCC
GCGACCTGATCCACTGGCGCCAGCTCCCGCTGGCCATGGTGCCCGACCAG
TGGTACGACACCAACGGCTTGTGGACGGGGTCCGCCACCACGCCTCCCCGA
CGGCCGCCTCGCCATGCTCTACACGGGCTCCACCAACGCCTCCGTCCAGG
TGCAGTGCCTGGCCGTGCCCGCCGACGACGCCGACCCGCTGCTCACCAAC
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Figure 5-1 The 5′ genomic sequence of *Ivr24* invertase. Regions shown include 978 bp of the promoter, the 1st, 2nd and part of the 3rd exon, plus the 1st and 2nd introns. Underlined areas designate, in 5′ to-3′ order, the ACGT cores, MotifIII like element (Ono et al. 1996), ACT cores, CE1 like element (Shen and Ho 1995), TATAA box, ATG start site, and the NDPNG consensus sequence for invertases. Introns are in bold italics.

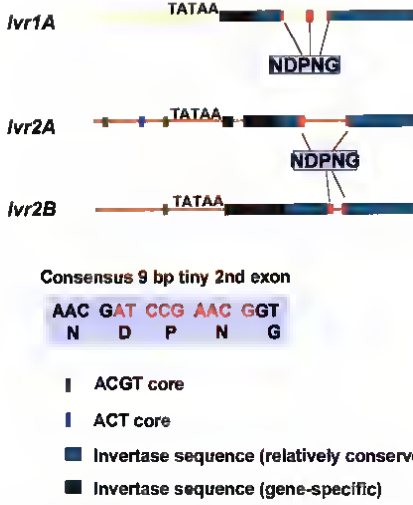

Figure 5.2 Diagramatic comparison of the 5' genomic sequences for maize *Ivr1A*, *Ivr2A* and *Ivr2B* soluble invertases. Note the contrast between intronic divisions of the NDPNG consensus sequence (catalytic site) of the *Ivr1A* vs *Ivr2* invertases. The *Ivr1A* pattern is typical of most invertases, which have their NDPNG domain divided by two introns that leave the smallest known plant exon between them. This encodes the core of the NDPNG. In contrast, both *Ivr2* genes lack this classic feature and are bisected by only one intron. The ACGT and ACT cores are also shown.

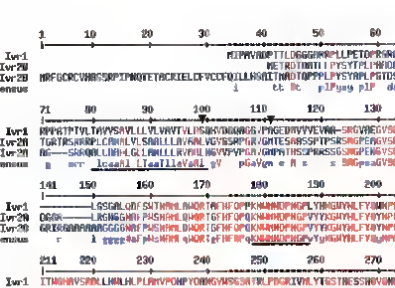

Figure 5-3 The alignment of N-terminal amino acid sequences from maize *Ivr1A*, *Ivr2A*, and *Ivr2B* soluble invertases. Analysis was done with the MultAlin program (Corpet 1988). Note that the consensus is much lower in the N terminal region. Red high consensus. Blue low consensus. Grey non-consensus. In the consensus line upper case represents homology among all three invertases whereas lower case represents homology between two of the three. The underline from position 80 to 100 shows a hydrophobic region which may function as a signal peptide, and the underline between 178 and 187 shows the region of greatest sequence similarity, including the NDPNG. The arrows at position 99 and 111 indicate possible cleavage sites for *Ivr1A* (99) and both *Ivr2A* and *Ivr2B* (111).

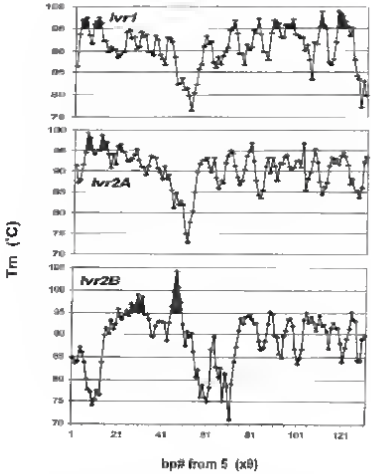

Figure 5-4 Tm curves of cDNAs for maize *Ivr1A*, *Ivr2A* and *Ivr2B* soluble acid invertases Tm values were calculated as per Breslauer et al (1986) with each point representing a mean from 36 nucleotides The distance between each point is 9 nucleotides Note the high Tm value (above 95°C) at the 5' end cDNAs of *Ivr2A* and *Ivr2B*

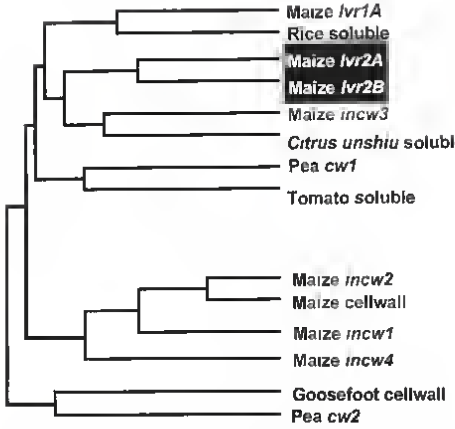

Figure 5 5 Phylogenetic analysis of soluble and cell wall invertases in vascular plants Invertases of maize and of other plant species were selected at random from the Genbank Analysis was done with the Blocks Multiple Alignment Processor copyrighted by Fred Hutchinson Cancer Research Center in Seattle, WA Note that the divergence of soluble and cell wall invertases appears to have occurred early in evolution prior to devergence of monocots from dicots

Table 5.2 Genes of plant invertases with or without the center of their NDPNG consensus sequence encoded by a 9 bp exon

	Species	Genes	Subcellular locations	Ref
Genes with 9 bp tiny exon	*Arabidopsis thaliana*	*Invertase2*	cell wall	Mercier and Gagarten 1995
	Daucus carota	*Inv*Dc2 Inv*Dc3*	cell wall	Lorenz et al 1995
		*Inv*Dc4 Inv*Dc5*	vacuole	Sturm 1996
	Lycopersicon pimpinellifolium	*InvLp6g*	vacuole	Elliott et al 1993
	Oryza sativa	*Inv1*	cell wall	Fernandes et al 1999
	Pisum sativum	*hfruct1*	cell wall	Zhang et al 1995
	Solanum tuberosum	*invertase*	cell wall	Bournay et al 1996
	Zea mays	*Ivr1*	vacuole	Xu et al 1995
		Incw1 Incw2	cell wall	Kim et al 1999
		Incw3 Incw4	cell wall	Taliercio et al 1999
Genes without 9 bp tiny exon	*Daucus carota*	*Inv*Dc1*	cell wall	Lorenz et al 1995
	Zea mays	*Ivr2A Ivr2B*	vacuole	Wu and Koch unpublished data

CHAPTER 6
FUNCTIONAL ANALYSIS OF UPSTREAM AND INTRONIC SEQUENCES OF SOLUBLE INVERTASE OF THE MAIZE *IVR1A* AND *IVR2A* AND *IVR2B* GENES

Introduction

Gene expression is regulated by interaction of *cis* and *trans* elements at levels of transcription, post-transcriptional processing, and longevity of mature mRNAs. Most of the regulatory information is contained in 5' promoter regions upstream of coding sequences. However, in eukaryotic cells, introns and 3' untranslated regions can also have regulatory roles (Gruss and Khoury 1980, Evans and Scarpulla 1989, Dean et al 1989, Vasil et al 1989, Clancy et al 1994, Fu et al 1995a, Fu et al 1995b).

For analysis of upstream regulatory elements and intron effects, transient expression assays have provided an effective and widely used approach, particularly for grain species (Herrera Estrella et al 1988, Ohta et al. 1990, Clancy et al 1994, Kao et al. 1996). Influence of test sequences can be quantified by fusing them to the coding sequence of a readily assayable reporter gene, such as β-glucuronidase (GUS, Jefferson et al 1987), chloramphenicol acetyltransferase (CAT, An 1986), β-galactosidase (LacZ, Matsumoto et al 1998), or green-fluorescent protein from jelly fish (Sheen et al 1998).

Although these constructs can be readily introduced into many dicot systems via various means (Draper et al. 1988), studies of monocots (including grains) has required

more extensive use of transient expression systems. Electroporation of leaf protoplasts has provided excellent information for short-term studies in maize (Sheen et al. 1993), whereas biolistic transformations of longer-lived cells have been important for longer-term responses (Kao et al. 1996).

Regulatory sequences in addition to those of the 5' flanking regions have received increasing attention (e.g. introns and 3' untranslated sequences) (Gruss and Khoury 1980; Evans and Scarpulla 1989; Dean et al. 1989; Vasil et al. 1989; Clancy et al. 1994; Fu et al. 1995a, Fu et al. 1995b). Regulation of gene expression by introns may involve alternative splicing patterns and interaction with transcriptional enhancers (Callis et al. 1987; Buchman and Berg 1988; Huang and Gorman 1990; Mascarenhas et al. 1990; Maas et al. 1990). In addition, the efficiency of intron splicing and splicing-dependent polyadenylation may be correlated with the magnitude of stimulated expression (Luehrsen and Walbot 1991; Clancy et al. 1994). The first intron from the maize *Sh1* sucrose synthase gene is one of the most actively studied introns. Its capacity to enhance gene expression up to 90 fold (Vasil et al. 1989) has made this intron particularly useful in commercial applications as well as investigations of gene expression in plant cells (McCarty et al. 1991, Hattori et al. 1992; Kao et al. 1996).

Soluble invertases are sugar- and cytokinin-responsive (Xu et al. 1996, Zeng et al. unpublished data). However, the mechanisms contributing to this regulation remain unclear. To date, cis-elements have been defined in promoters of sugar-responsive genes but consensus sequences have not yet emerged among them (see Table 5-1). Several sugar repressive elements have been identified in photosynthetic genes upstream of their TATA boxes, but again, these do not share consensus features with sugar responsive

elements identified thus far in other organisms (Sheen 1990). 1MH2, a 16 bp sequence in the promoter of maize synthase from cucumber was found to be essential for sugar induced responses (Sarah et al 1996). An element conferring a strong sucrose repression was also identified in the -205 to -187 region of the promoter for rbcS2 in *Phaseolus vulgaris* (Urwin and Jenkins 1997). This sucrose repression region contains a G-box (CACGTG) located between positions -205 and -200, plus two other sequences resembling a SUcrose Responsive Element (SURE) from potato (Grierson et al 1994) and a Carbohydrate Responsive Element (ChoRE) in mammalian genes (Kaytor et al 1997). In addition, induction of the rice *Amy3D* amylase by sugar depletion was found to require a 50 bp nucleotide sequence in the promoter region between positions -172 and -123 (Toyofuku et al 1998). Thus far, these studies have not identified consensus sequences for cis-elements responding to sugar signals in plants, although numerous investigations have indicated that an ACT core may be central to this function (Ishiguro and Nakamura 1992, Grierson et al 1994, Kaytor et al 1997).

The purpose of the work presented here was two fold. The first was to test the degree to which sugar and cytokinin responsiveness of the soluble invertases in maize was mediated by 5' upstream promoter sequences. The second was to determine whether leading introns from the same invertases, or known regulatory introns from other genes, could alter sugar or cytokinin responses of the *Ivr1A* or *Ivr2A* invertases.

Materials and Methods

Construction of GUS Reporter Genes

All chimeric-gene constructs were cloned in the *E. coli* plasmid pUC19. Correct orientation and order of gene elements were verified by analysis of restriction fragments and DNA sequences. Constructs lacking the maize *Sh1* first intron were generated using the commercially available pBI221 (Clontech, Palo Alto, CA) which contains a modified CaMV 35S promoter, a GUS coding sequence and a NOS 3' polyadenylation signal. The 35S promoter was replaced with various invertase-promoter elements in the final constructs. Constructs containing the maize *Sh1* first intron were synthesized using a D40.1 plasmid (Kao et al. 1996) in which a maize *C1* basal promoter, *Sh1* first intron, GUS coding sequence, and NOS 3' polyadenylation signal were sequentially cloned in a pUC19 plasmid. The *C1* basal promoter was substituted with test sequences from the *Ivr1A* soluble invertase gene.

For construction of *Ivr1A* + GUS chimeric genes, PstI and BamHI restriction sites were introduced via PCR primers at either end of the *Ivr1A* promoter region to be tested, at -676 and -1, respectively (Figure 6-1A). The resulting PCR product was digested with PstI and BamHI and purified with Wizard PCR Preps DNA Purification System (Promega, Madison, WI). Plasmid pBI221 was digested with PstI and BamHI to remove the CaMV 35S promoter, and the fragment containing the GUS coding sequence was purified and ligated with the *Ivr1A* promoter (Figure 6-1A). Sequences of PCR products were verified by sequence analysis.

The construct containing the *Ivr1A* first intron, in addition to upstream regulatory sequences (*Ivr1A* + *Ivr1A* intron + GUS) was synthesized in a similar way except that a BamHI site was introduced at a point +772 into the *Ivr1A* coding sequence rather than at the –1 position. The construct contained the first exon (236 bp), first intron (458 bp) and the first 5 nucleotides of second exon (9 bp total for exon). After splicing GUS and the *Ivr1A* test sequences were in the same reading frame.

Where the *Sh1* first intron was substituted for that of *Ivr1A* (*Ivr1A* + *Sh1* intron + GUS) KpnI and XbaI sites were introduced respectively into positions -676 and -1 upstream of the coding sequence. The resulting PCR product and plasmid D40-1 (Kao et al. 1996) were both digested with KpnI and XbaI followed by purification and ligation of corresponding fragments.

The construct containing the *Cat1* first intron (*Ivr1* + *Cat1* intron +GUS) was synthesized from plasmid pIG221 (Ohta et al. 1990), which contains a *Cat1* first intron upstream of the GUS coding sequence. Plasmid pIG221 was digested with BamHI, followed by filling the overhang with the Klenow fragment of DNA polymerase and digestion with PstI. The *Ivr1A* promoter from position -676 to -1 was digested with BalI (resulting in a blund end) and PstI. This *Ivr1* promoter sequence was ligated upstream of the *Cat1* intron.

Constructs using the *Ivr2A* promoter were produced in a similar manner to those with the *Ivr1A* promoter. For *Ivr2A* + GUS, the *Ivr2A* promoter fragment between positions -925 and -32 was amplified via PCR, and HindIII and XbaI sites were introduced to upstream and downstream ends, respectively. This fragment was also used to build the *Ivr2A* + *Cat1* intron + GUS construct. The *Ivr2A* + *Ivr2A* intron + GUS

construct contains *Ivr2A* genomic sequence from position -725 to +1028 (including the first and second exons plus the first and second introns of *Ivr2A*) HindIII and XbaI sites were also introduced into the ends of this fragment. The *Ivr2A* + *Sh1* intron + GUS construct has the same promoter fragment as that of the *Ivr2A* + GUS construct, but also has an additional KpoI site instead of a HindIII site at the 5 end

For building the construct containing the leading introns of *Ivr2A*, an Advantage-GC PCR Kit (Clontech, Palo Alto CA) was used to introduce restriction sites at points flanking the promoter and 5 coding regions This kit effectively opened GC rich regions at 95°C whereas this result was not obtained using other commercially available materials

Biolistic Transformation

The plasmid DNA/gold mixture and the bombardment parameters were as described by Kao et al (1996) The plasmid was prepared by mixing 37 µl of 40 mg/ml gold stock solution (1 6 µm diameter, Biorad Inc., Hercules, CA) in a 500 µl Eppendorf tube then sequentially adding 25 µl water 5 µg *Ivr1A* promoter/GUS chimeric DNA and 5 µg internal control plasmid DNA (a ubiquitin promoter driving a luciferase coding sequence) After briefly vortexing this preparation 20 µl of 100 mM free base spermidine and 50 µl of 2.5 *M* $CaCl_2$ were placed in separate drops on the side of the tube to avoid premature mixing of either solution with the DNA or gold particles DNA was precipitated and attached to the gold particles by immediately mixing the solutions and vortexing the tubes for 20 s. The tubes were centrifuged for 2 s and the supernatant

removed. Tubes were sonicated for a few seconds after adding 200 µl of 100% ethanol. The supernatant was discarded after briefly centrifuging to pellet the DNA/gold mixture. Tubes were again sonicated for several seconds after adding 60 µl of 100% ethanol, then kept on ice. A 5-µl final volume of finally prepared DNA/gold mixture was used for each biolistic transformation.

Extraction and Quantification of Transient Expression

GUS and luciferase activities were assayed after biolistically transformed cells had been incubated for 18 to 20 h. Transiently transformed cells were ground in a pre-chilled mortar with 0.3 ml of extraction buffer (0.1 M potassium phosphate pH 7.8, 2 mM EDTA, 2 mM DTT, 5% v/v glycerol) and a small amount of carborundum to facilitate grinding. The ground mixture was transferred to a 1.5 ml Eppendorf tube and centrifuged at 14,000 rpm for 8 min at 4°C. Supernatant was transferred to a new Eppendorf tube and kept on ice for GUS and luciferase assays.

GUS activities were assayed using the method previously described by Jefferson et al. (1987). Reaction substrate buffer was prepared by adding 1 mM MUG (4-methyl umbelliferyl-β-D glucuronide, Sigma M9130) in GUS extraction buffer. For assays, 50 ml of extract was added to 75 ml of this GUS reaction buffer. The reaction mixture was incubated at 37°C for 20 min or 2 h, after which 50 µl samples were immediately added to 1 ml stop buffer (0.2 M Na_2CO_3). For GUS quantification, 800 µl of the 1 ml stop buffer with sample was diluted 4.75 times by adding an additional 3.8 ml of stop buffer. Luciferase activities were quantified by mixing 20 µl of the cell extract with 200 µl of ice

cold reaction buffer (25 mM tricine pH 7 8 15 mM MgCl2 5 mM ATP, 0 05% w/v SA) in a reaction tube (12 x 75 mm) and assayed using a Monolight Luminometer (Analytical Luminescence Laboratory) (Kao et al., 1996) Insertion of the tube into the luminometer resulted in automatic injection of 100 µl of substrate buffer (1 mM luciferin pH 9 5). Emitted photons were counted for 10 s and activities recorded as standard Relative Light Units (RLU)

Plant Materials

Maize cell culture (BMS) was regularly maintained in MS medium containing 3% sucrose (ca. 90 mM) and 2 µg/ml 2, 4-D (both standard for cell culture) at room temperature with moderate agitation The cultured cell line was transferred to fresh medium once per week For transient expression assays cells were washed briefly with sugar and 2 4-D-free MS medium and evenly distributed on 2 5 cm filter paper disks

Results

Initial analyses of transient expression indicated that activity of the *hvr1A* promoter was only modestly affected by changes in sugar and kinetin availability (data not shown) The latter amounted to only ca. 10% In these first experiments, the primary test construct consisted of only an *hvr1A* promoter + GUS reporter, and a study period of limited-duration. Following bombardment, cells were cultured on solid MS media 1) without sucrose, 2) with 3% sucrose or 3) without sucrose but with 2 0 µM

kinetin. GUS and Luciferase activities were assayed after a 20-h incubation at room temperature in the dark. The results were significantly less marked than would have been predicted on the basis of previous northern blots (Zeng et al. 1999). One possible reason for this difference was considered to have been that of treatment duration, which was significantly longer (24 h to 48 h) when sugar and kinetin responses that were analyzed by northern blot.

To test if the less pronounced effect of sugar and kinetin on the *Ivr1A* promoter was due to a limited duration of exposure, cultured cells were transferred to experimental media both before and after biolistic transformation. Media for pre-incubation consisted of MS medium 1) without sucrose or kinetin, 2) with 3% sucrose (ca. 90 mM) but no kinetin, 3) without sucrose but with 2 µM kinetin, or 4) with 3% sucrose and 2 µM kinetin. Cells were transferred to these pre-treatments for 24 h and 48 h before being evenly distributed on filter paper discs for bombardment.

Figure 6-2 shows that when a 24-h pre-incubation was included, distinct responses to cytokinin were observed that differed depending on sucrose availability. The longer period of total exposure to experimental conditions appeared to be essential for responses to sugars and cytokinins at the promoter level. Results of experiments conducted in this way demonstrated sugar and kinetin responses of a magnitude compatible with previous northern blot analysis. In all instances, transient expression analysis of the *Ivr1A* promoter-GUS construct showed approximately 20% - 100% higher activity with 3% sucrose (ca. 90 mM) than when sucrose was depleted. Responses to 2 µM kinetin also showed from 50% to 100% increases in *Ivr1A*-GUS activity with and without sucrose, respectively. Similar results were obtained from cells after a 48-h pre-

incubation (data not shown). This evidence, together with other results (see following text), supports the suggestion that cytokinins may act, at least partially, at a transcriptional level for the *Ivr1A* promoter.

The *Ivr1A* promoter alone enhances reporter activity only two- to three-fold above that of the basal promoter (a 35S TATA control). This activity is not sufficient for further dissection of promoter function. In previous work, introduction of the maize *Sh1* first intron (Kao et al. 1996) or the castor bean *Cat1* first intron (Ohta et al. 1990) successfully increased the respective activity of the maize *C1* and CaMV 35S promoters to multiple levels above those of intronless constructs. To test whether the first intron of the *Ivr1A* maize invertase had a similar function, this intron was introduced into an *Ivr1A* + GUS construct to form a new construct designated "*Ivr1A* + *Ivr1A* intron + GUS". Transient expression of these two constructs were compared in the presence and absence of 3% sucrose. At either low (0%) or high (3%) sugar levels, the construct with the *Ivr1A* intron showed either slightly lower or similar activity to that of the intronless construct (Figure 6-3). These results indicate that the *Ivr1A* first intron did not markedly affect its own gene expression.

In order to obtain the greatest possible activity in *Ivr1A* promoter constructs and also to test regulatory effects of foreign introns, the maize *Sh1* first intron and the castor bean *Cat1* first intron were inserted separately into *Ivr1A* + GUS constructs between the *Ivr1A* promoter and GUS coding sequences. These two new constructs were designated *Ivr1A* + *Sh1* intron + GUS and *Ivr1A* + *Cat1* intron + GUS, respectively. Activities of all four *Ivr1A* promoter constructs were tested either at different sugar levels (Figures 6-3 and 6-5A) or at different kinetin levels (Figure 6-4A). In both experiments, constructs

with the *Sh1* intron showed a three to four fold increase in activity compared with the intronless *Ivr1A* + GUS. Results of a fourth construct showed that although the *Cat1* intron was previously found to have a positive effect in conjunction with the 35S promoter, it had a negative effect when combined with the *Ivr1A* promoter. In addition, a marked difference was evident in responses to media with or without sucrose when the *Cat1* intron was present. However, the *Ivr1A* promoter + *Cat1* intron construct was able to drive only about half as much the GUS reporter activity as was the *Ivr1A* promoter alone.

For the results shown in Figure 6-5A, the extent of sucrose deprivation was altered to determine whether a sucrose-free medium represented too severe a treatment. If so, then metabolic consequences could have been great enough to affect the accuracy of the internal control (luciferase activity driven by the ubiquitin promoter), which is the basis for calculation of relative GUS activity. This possibility was addressed by adding 0.3% sucrose (ca. 9 mM) to the sugar-depletion treatments. Activities of all four *Ivr1A* promoter constructs at the low sugar level (0.3% sucrose) were significantly increased compared to those without sucrose (refer back to Figure 6-3). Results indicated that a minimal sucrose level was needed for maintaining stable metabolism, which in turn provided a reliable internal control. However, although analysis of mRNA abundance showed higher levels of *Ivr1A* invertase mRNA under similar low-sugar conditions (Zeng et al. 1999), only the construct with the *Ivr1A* intron clearly responded in this fashion in the present study (Figure 6-5A).

Responses of the *Ivr2A* promoter were also tested in this system (Figure 6-4A and Figure 6-5B). Cultured cells were preincubated for 24 h in 2,4D-free MS media

containing 0.2% glucose either with or without 2 μM kinetin. As observed for the *Ivr1A* promoter, the *Ivr2A* construct with a *Sh1* intron (*Ivr2A* + *Sh1* intron + GUS) showed greater expression than the other three constructs, regardless of kinetin availability. Cytokinin increased mean GUS activity by ca. 30% for the *Ivr2A* + *Sh1* intron + GUS construct (Figure 6-4B). The other three constructs did not show significant differences in responses to kinetin. The extent to which kinetin enhanced expression of the *Ivr2A* + *Sh1* intron + GUS construct approximated that observed for mRNA levels in whole plant and organ systems (Zeng et al., unpublished data). In contrast to the minimal influence observed for the *Ivr1A* intron on its own promoter activity, constructs with the *Ivr2A* intron (*Ivr2A* + *Ivr2A* intron + GUS) showed 100% elevations in GUS activity compared to intronless constructs regardless of kinetin status. The same was not observed for constructs with a *Cat1* first intron, which appeared to have a negative effect on the *Ivr2A* promoter.

To test the sugar responsiveness of the *Ivr2A* promoter, transient expression of the *Ivr2A* promoter/GUS reporter gene constructs was quantified at 0.3% and 3.0% sucrose levels. Cultured cells were transferred to 2,4D-free MS medium containing either 0.3% or 3.0% sucrose 24 h before biolistic transformation. The *Ivr2A* promoter showed 30% to 40% greater activity at elevated sucrose concentrations (3.0%) than at lower sugar levels (0.3%) (Figure 6-5B). As observed above for the *Ivr1A* promoter, *Ivr2A* constructs with the *Sh1* first intron showed the highest activity among all the four *Ivr2A*/GUS constructs. Interestingly, the construct containing the *Ivr2A* intron also showed about 100% higher activity in the presence of abundant sucrose compared with its intronless counterpart. The positive effect of the *Ivr2A* intron on its own gene expression is thus especially

evident in plus-sugar treatments. Again, a fourth construct showed that despite the previously reported capacity for the *Cat1* first intron to enhance gene expression with the 35S promoter (Ohta et al. 1990), the negative effect of this intron in combination with the invertase promoter extended across sugar and cytokinin treatments.

Discussion

Numerous efforts have been directed toward analysis of 5' upstream promoter regions mediating sugar-responsive gene expression (Sheen 1990; Grierson et al. 1994; Sarah et al. 1996; Urwin and Jenkins 1997; Toyofuku et al. 1998), but evidence remains fragmentary. Even less is known about cytokinin regulation at the promoter level. Intron-enhanced gene expression is evident in many instances (Vasil et al. 1989, Luehrsen and Walbot 1991; Clancy et al. 1994), although the detailed mechanism remains undefined. Evidence presented here indicated that both promoters and introns are involved in regulation of sugar- and cytokinin-modulated invertase expression.

Sugar- and cytokinin-induced expression of soluble invertases was evident only when treatments began prior to transformation (Figure 6-2), probably because of the time involved in the change of sugar and metabolic status of cultured cells. Not only are responses of cell cultures typically slower than those of apical meristems or isolated protoplasts, but sugar responses of many genes occur over a relatively extended period of time. The extent of increased GUS activity in response to sugar and kinetin is similar to that observed for mRNA levels under similar conditions and over a similar timespan (Zeng et al. 1999; Zeng et al. unpublished data). In both instances, 12 to 24 h are needed,

effectively for responses by soluble invertase genes to signals transduced from sugars or cytokinins. A series of protein kinase/phosphatase cascades appears to be involved (Jang et al. 1997; Koch et al. 1999; Sturm 1999; Sheen et al. 1999). Results in Figure 6-2, together with those of Zeng et al. (1999), suggest a distinct period less than 24 h is required between initiation of sugar signals and responses by specific genes.

Although the first intron of the *Ivr1A* gene did not enhance overall expression for the constructs tested in the present study (Figures 6-3, 6-4A, and 6-5A), regulatory elements for the sugar-depletion response were not examined and could reside in this intron. In fact, only the construct with the *Ivr1A* first intron showed enhanced activity when sugar was depleted (Figure 6-5A). The possibility also remains that this intron could regulate *Ivr1A* invertase expression under certain conditions not tested or among different cell types in intact plants. Recent work has shown that introns can also modify patterns of gene expression among different cells (Sieburth and Meyerowitz 1997), in addition to their potential involvement in alternate splicing and polyadenylation processes (Luehrsen and Walbot 1991; Collis et al. 1990; Huang and Gorman 1990).

The maize *Sh1* and castor bean *Cat1* first introns apparently did not change the perception of sugar signals by the *Ivr1A* promoter because these two introns did not have a similar effect to that of the *Ivr1A* first intron on sugar responses (Figure 6-5A). Although the overall activity was altered significantly in the presence of these two introns, the extent to which the *Ivr1A* promoter responded to sugar availability remained nearly unchanged (Figures 6-3 and 6-5A). The degree to which the *Sh1* intron enhanced expression of the *Ivr1A* promoter (Figures 6-3, 6-4A, and 6-5A) was substantially less than reported for other gene promoters examined thus far (Vasil et al. 1989; Clancy et al.

1994 Hattori et al 1992, Kao et al 1996) Three- to four-fold increases were evident in the present study vs. ten- to hundred-fold in combination with other promoters

The castor bean *Cat1* first intron apparently does not coordinate effectively with the *Ivr1A* promoter for direct reportor gene expression (Figures 6-3 6-4A and 6-5A) Although this intron can dramatically enhance GUS gene expression driven by the CaMV 35S promoter (Ohta et al 1990 Mitsuhara et al 1996) it had little effect when in conjuction with the *Ivr1A* promoter Expression was even reduced in some instances It is not clear why the *Cat1* intron has a negative effect when together with the *Ivr1A* promoter in particular However these results suggest that cooperation between promoter and intron may be essential for intron enhancement of expression

The *Ivr1A* invertase promoter showed a distinct transcriptional component in response to cytokinin, especially when sugar was depleted (Figures 6-2 and 6-4A) This result was consistent with previous analyses of mRNA abundance (Zeng et al unpublished data) The *Sh1* intron increased the *Ivr1A* promoter/GUS expression in response to kinetin by 3-fold, whereas the effect of the *Ivr1A* intron and *Cat1* intron was much less evident (Figure 6-4A)

This result is significant due to the few known instances of cytokinin-responsive gene expression that operate at a transcriptional level. Most reported cytokinin-regulated gene expression appears to be mediated primarily at a post-transcriptional level (Suzuki et al 1994, Crowell 1994 Silver et al 1996 Downes and Crowell 1998) Cytokinin enhanced stability of mRNAs at a post-transcriptional level possibly involves newly synthesized proteins (Suzuki et al 1994) and/or protein phosphatase (Downes and Crowell 1998) Our result showed that at least part of cytokinin-induced *Ivr1A* invertase

expression may be regulated at the transcriptional level. Unfortunately, further dissection of responsive elements requires marked changes of expression (at least 10 fold and typically 100 fold [for example Tanaka et al 1990 Matsuhara et al. 1996, Kao et al 1996]). Many physiologically significant responses however, involve less prominent changes, such as the 50% enhancement observed in the present study and in analyses of other changes in mRNA abundance.

Cytokinin-regulated expression of *Ivr2A* invertase was only observed with the construct containing the *Sh1* first intron (*Ivr2A* + *Sh1* intron + GUS) possibly because expression levels were great enough to distinguish significant differences (Figure 6-4B) The extent of enhanced *Ivr2A* expression in response to cytokinin was much less than that of *Ivr1* (Figure 6-4A), consistent with mRNA abundance analyses (Zeng et al unpublished data) Levels of *Ivr1A* mRNA and enzyme activity indicated that this gene was more sensitive to cytokinin than was *Ivr2A*, but both responded to this growth regulator We can not conclude at which level (transcriptional or post-transcriptional) *Ivr1A* and *Ivr2A* invertases are upregulated by cytokinin from current data. However, it is clear that up stream sequences are involved in both instances

Results of transient expression analysis using a GUS reporter gene driven by the upstream *Ivr2A* promoter were compatible with the sugar-responsiveness of this gene in intact plant systems (Figure 6-5B) The extent of sugar induced expression in transient analysis corresponded to that observed from analysis of mRNA abundance (Zeng et al 1999) In the present study relative promoter activity was about 30% greater when sugars were abundant than when they were limited Although expression of the *Ivr2A* promoter GUS construct increased by about 2-fold with addition of a *Sh1* first intron, the

extent of sugar responsiveness remained unchanged (Figure 6 5B) The ACGT and ACT cores of putative response elements upstream of *Ivr2A* may have contributed to this induced activity (see discussion in Chapter 5) However the difference between promoter activity at high and low sugar levels was insufficient for reliable dissection of responsible domains

Unlike the *Ivr1A* first intron, leading introns of *Ivr2A* can effectively increase GUS expression by more than 100% (Figure 6-4 Figure 6 5) This indicates that introns of *Ivr2A* can positively regulate expression of their own gene inside plant cells It is possible that distinct regions or elements of control may lie within *Ivr2A* introns as hypothesized for the *Sh1* first intron (Clancy and Hannah unpublished data) and both the extent and patterns of expression may be altered as for Sieburth and Meyerowitz (1997) This question will be further addressed using stable transgenic maize plants expressing an *Ivr2A* promoter driving a GUS reporter with or without leading introns of *Ivr2A* Constructs prepared and tested in transient expression systems in the present study now justify a thorough analysis of *Ivr2A* and *Ivr2A* + its own intron in stably transformed maize plants These two constructs were provided to Pioneer Hi Bred Inc (Johnston Iowa) for introduction into such transgenic plants and seeds have now been returned for analysis

Figure 6 1 Constructs used for transient expression assays. (A) Flow chart demonstrating the procedure for synthesis of the experimental *Ivr1A*+GUS construct (B) Diagramatic representation of the four different constructs used for transient expression assays in this chapter

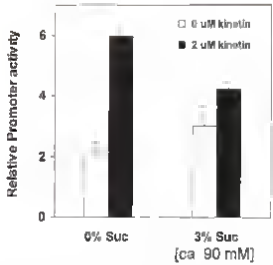

Figure 6-2 Relative activity of the *Ivr1A* promoter in response to sucrose or kinetin Maize suspension cells (BMW) were cultured in MS medium containing 3% sucrose and 2 μg/ml 2 4 D (Both are standard for cell cultures) at 22°C with moderate agitation (120 rpm) Cultured cells were transferred to fresh medium each week For transient expression assays cultured cells were transferred from standard MS medium to experimental solution for pre-incubation The latter consisted of MS medium 1) without sucrose or kinetin, 2) with 3% sucrose (ca 90 mM) but no kinetin, 3) without sucrose but with 2 μM kinetin, or 4) with 3% sucrose and 2 μM kinetin Cells were pre-incubated for 24 h before being evenly distributed on filter paper discs for biolistic transformation The *Ivr1A* + GUS construct was used to test promoter activity of the *Ivr1A* invertase gene alone Following biolistic transformation, cells were cultured on solid MS Media with the same composition used for pre-incubation. GUS and Luciferase activities were assayed after 20 h of incubation at 22°C in the dark Error bars denote SEMs of three different experiments each using different sets of biolistically transformed maternal

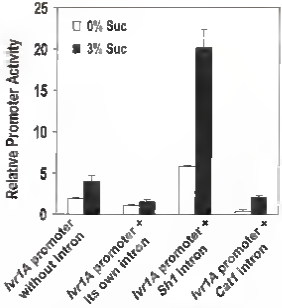

Figure 6-3 *IvrIA* promoter construct activities in cell suspension cultures with and without 3% sucrose (ca. 90 mM standard for cell culture) Cultured maize cells were pre incubated in MS medium with or without 3% sucrose for 24 h The four constructs included the 5 promoter region from -676 bp to -1 bp upstream of the coding sequence either alone with the GUS reporter (*IvrIA* + GUS) or with additional intron sequences from the *Ivr1* invertase (+1 bp to +772 bp of *IvrIA* [*IvrIA* + *IvrIA* intron + GUS] or the first intron of *Sh1* sucrose synthase [*IvrIA* + *Sh1* intron + GUS]), or the first intron of the Cat1 catalase ([*IvrIA* + *Cat1* intron + GUS]) GUS and Luciferase activities were assayed after 20 h of incubation at 20°C in the dark Error bars denote SEMs of three different experiments each using different sets of biolistically-transformed material

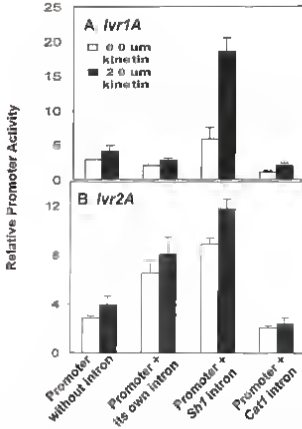

Figure 6-4 Influence of kinetin (2 µM) on promoter-construct activities for **A.** *Ivr1A* and **B** *Ivr2A* soluble invertases Cultured maize cells were pre incubated in MS medium containing 3 0% sucrose (ca 90 mM, standard in cell culture) with or without 2 µM kinetin for 24 h The eight constructs included the 5 promoter region upstream of the coding sequence, either alone with the GUS reporter or with additional leading-intron sequences from the *Ivr1A* or *Ivr2A* gene the *Sh1* first intron or the *Cat1* first intron (see materials and Methods for detail) GUS and Luciferase activities were assayed after 20 h of incubation at 22°C in the dark. Error bars denote SEMs of three different experiments each using different sets of isolistically-transformed material

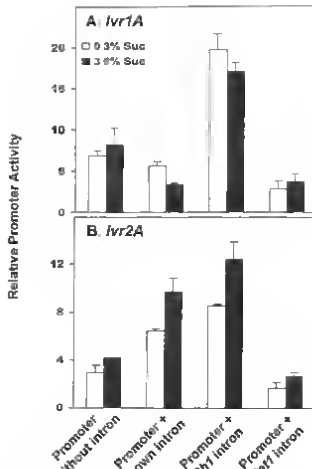

Figure 6-5 Influence of sugar availability at limiting (0.3% sucrose [ca. 9 mM]) or abundant (3.0% sucrose [ca. 90 mM]) levels on promoter-construct activities for **A.** *Ivr1A* and **B.** *Ivr2A* soluble invertases. Cultured maize cells were pre-incubated in MS medium with either 0.3% or 3.0% sucrose for 24 h. The eight constructs included the 5' promoter region upstream of the coding sequence, either alone with the GUS reporter or with additional leading intron sequences from the *Ivr1A* or *Ivr2A* gene, the *Sh1* first intron, or the *Caf1* first intron (see materials and Methods for detail). GUS and Luciferase activities were assayed after 20 h of incubation at 22°C in the dark. Error bars denote SEMs of three different experiments each using different sets of biolistically-transformed material.

CHAPTER 7
SUMMARY AND CONCLUSIONS

Sucrose occupies a vital position in plant growth and development, because it is the principle photosynthetic product transported via phloem from source to sink tissues Invertase and sucrose synthase (reverse reaction) catalyze the only known enzymatic means of sucrose cleavage in plant cells These two enzymes are thus pivotal in the control of carbohydrate partitioning and utilization in most vascular plants (Avigad 1982 Turgeon 1989 Duke et al 1991 Koch et al 1996) More recently this centrality has also been found to include a role in sugar regulated gene expression (Koch 1996, Jang et al 1997 Sheen et al 1999 Sturm 1999 Koch et al, 2000) since invertases provide more substrates for hexose-based sugar "sensing" system than does sucrose cleavage via the reversible sucrose synthase reaction In addition both invertase and sucrose synthase genes are sugar responsive (Koch et al 1992 Xu et al 1996 Koch et al 1998) Finally localized changes in sucrose synthase expression have been observed in response to sugar availability and suggest possible roles in partitioning resources among individual cells (Koch et al 1992) If patterns of invertase expression also change in response to sugar signals, then this could provide a mechanism not only for further enhancement of import, but also for localized amplification of sugar signals to responsive genes in key cells

One of the primary hypotheses tested here was that a central feature of sugar responsive change in soluble invertase gene expression was a shift in localization at a cell

and tissue level. In each instance projected sites of change are those known to play key roles in organ development and/or sucrose import. If so, then this evidence could provide an important basis for interpreting changes in gene expression in a physiological context.

To accomplish this goal, an *in situ* hybridization approach was utilized to localize mRNAs of the *Ivr2* soluble acid invertase within individual cells of maize ovules, young kernels, and in root tips. Responses to developmental perturbations were examined first. Previous analyses had indicated up-regulation of soluble invertases at the mRNA and enzyme activity levels in response to pollination (Xu et al. 1996) and a potentially important role for these enzymes in kernel set under stress (Chetkh and Jones 1994, Zinselmeier et al. 1999).

In ovules and young kernels, the period immediately before and after pollination was characterized by the presence of *Ivr2* mRNAs primarily in vascular tissues and pericarp. Pollination induced a sharp, localized rise in this *Ivr2* expression. Cells of the pericarp and nucellus are undergoing rapid expansion during the earliest stage of kernel development (Kiesselbach 1949). However, enlarging cells in the upper ¼ of the nucellus were not among sites where *Ivr2* mRNA was abundant. At 4 to 7 days after pollination, the embryo-endosperm complex joins the pericarp and nucellus as a rapidly expanding structure, and eventually dominates sucrose import into the kernel (Kiesselbach 1949). During this period, *Ivr2* mRNA was localized primarily in the pericarp and pedicel region, but was also evident in specific cells of the endosperm/embryo complex. The *Ivr2* soluble acid invertase was expressed only during a narrow window of the post pollination period (less than 10 days) during which kernel set was determined. A post pollination burst in expression was localized to nutritive

maternal tissues which expanded rapidly at this phase in development, consistent with their proposed role in sink establishment and early kernel set.

In root tips of 4- or 5 day-old maize seedlings the *Ivr2* soluble invertase mRNA was primarily expressed in epidermis, vascular bundles, cortex, and inner stele. Each of the four regions is similar in having a potentially elevated demand for sugars and/or undergoing active expansion (Kuiper 1949, Weier et al. 1974). Elevated expression of soluble invertase in epidermis may concurrently provide hexoses for carbohydrate based expansion as well as ATP utilized during the active, energy consuming process of nutrient uptake. In and near the vascular tissues of root tips, soluble invertases are likely to promote the symplastic unloading which predominates in these organs (Bret-Harte and Silk 1994). Soluble invertase may also play a role in expansion during development of vascular cells. Expression of soluble invertases within cells of the cortex and inner stele may contribute hexoses to cell turgor, which in turn can support root elongation (Bengough et al. 1997, Frensch and Hsiao 1994).

The profile for expression of the *Ivr2* soluble acid invertase at the cell level changed with perturbation of glucose and cytokinin availability. These corresponded to changes in structure of the root apex and extent of root tip elongation. When carbohydrates were depleted, soluble invertase mRNAs were essentially restricted to epidermal regions, and to some degree the stellar zone. This expression pattern could prioritize use of limited C-resources to the most essential tissues as well as limit C contributions to osmotic constituents of expanding cells in the cortex. In contrast, when sugars were abundant, the *Ivr2* soluble invertase mRNA was expressed at relatively similar levels throughout the root cells proximal to the 2 mm of root tips, which could

facilitate carbohydrate import and turgor maintenance during elongation and expansion of these cells (Beaugough et al. 1997, Frensch and Hsiao 1994). Patterns of *Ivr2* soluble invertase expression also changed in response to kinetin treatments, but at cell and tissue levels these resembled mRNA localizations observed under carbohydrate deprivation. There was minimal label in zones likely to contribute more prominently to root tip elongation, such as the cortex and inner stele. Thus, despite kinetin-enhancement of *Ivr2* mRNA levels, this was not reflected in root tip elongation.

A second goal of the research presented here was to test the degree to which soluble invertase expression was associated with cell expansion. The maize root-tip system has been widely used in previous studies of cell expansion (Webster et al. 1991, Sharp et al. 1994) and has been well characterized in this regard. Extent of carbohydrate based differences in elongation rate corresponded well to soluble invertase expression at both the mRNA and activity levels. However, kinetin enhanced soluble invertase activity without concurrent elongation of root tips, and sites of *Ivr2* mRNA localization did not appear likely to contribute to elevated invertase activity in zones of lateral expansion. Further analysis of possible ethylene involvement in this kinetin effect revealed that both an inhibitor (AVG) and precursor (ACC) of ethylene biosynthesis could independently alter root elongation and/or soluble invertase expression. Root tip elongation was thus related to timing, sites, and extent of *Ivr2* soluble invertase expression during carbohydrate-based differences in growth, but not when elongation was modulated independently of carbohydrate supplies.

A third goal of this work was a molecular characterization of the soluble invertases in maize. Two *Ivr2* genomic sequences were isolated and designated *Ivr2A*

and *Ivr2B* respectively Sequence analysis showed that these invertases differed from most others, including *Ivr1A* in their lack of intron divisions within the high conserved NDPNG motif Other invertases typically have the three central amino acids (DPN) of their consensus NDPNG sequence isolated on a small, 9 bp exon. Instead, *Ivr2A* and *Ivr2B* have nearly intact NDPNG sequences encoded at the end of their second and first exons, respectively A distinct feature of *Ivr2A* is that it appears to have a very tiny first exon (40 bp) and very small first intron The first two thirds of the second exon shares no similarity with any known invertase sequences The derivative amino acid sequence of the last one third of this exon shows a high degree of similarity about 70% to the first exon of many other invertases In *Ivr2B* the intron following the NDPNG invertase consensus sequence has only 82 bp and is thus substantially smaller than that of other known invertase introns at this position. A blast search revealed that the first half of the first exon shows no similarity whereas the second half has 68% identity to derivative amino acid sequences of the othel invertases All three soluble invertase genes were found to have an extremely GC-rich region near the 5 end of their coding sequences. Resulting Tm values for these domains typically rose well above 94°C. the temperature used for opening double DNA chains during standard PCR based procedures The Tm values of *Ivr2A* and *Ivr2B* were 5 to 10 degrees higher than for *Ivr1A* Phylogenetic analysis of soluble and cell wall invertases from varies species indicated that evolutionary divergence between soluble and cell wall invertases occurred early, and that the two subgroups of soluble invertases (*Ivr1*- and *Ivr2*-like) also diverged relatively long ago

A fourth goal of this work was to test the degree to which regulation of maize soluble invertases by sugars or cytokinins involved transcriptional control and/or intron

effects. Toward this end, a series of chimerical genes was constructed with promoters of maize soluble acid invertases driving a GUS reporter. Maize cell cultures were used for analysis of transient expression. Soluble acid invertases in maize, especially *Ivr1* can be up-regulated by cytokinins (Zeng et al. unpublished data), suggesting that cytokinins may have a positive effect on initiating sugar signals. Previous work has indicated that cytokinins primarily regulate genes at a post transcriptional level (Crowell 1994; Silver et al. 1996; Downes and Crowell 1998). However, analysis of the *Ivr1* soluble acid invertase promoter showed higher activity in the presence of kinetin, especially at low sugar levels. These results were consistent with previous northern blot analysis, and further indicated that up-regulation of maize soluble acid invertase by cytokinins occurred at least partially at a transcriptional level.

In order to test effects of intronic sequences on transcriptional regulation of the *Ivr1A* and *Ivr2A* invertases, the *Ivr1A* and *Ivr2A* first introns, maize *Sh1* first intron, and castor bean *Cat1* first intron were inserted separately between invertase promoters and GUS coding sequences. Constructs with the *Sh1* intron showed a three- to five-fold increase in activity compared with the intronless *Ivr1A* + GUS or *Ivr2A* + GUS. Although the *Cat1* intron had been demonstrated to have a positive effect with the 35S promoter in previous studies (Ohta et al. 1990), it had a negative effect when combined with the *Ivr1A* promoter. Although the *Ivr1A* first intron did not significantly affect its own gene expression, the leading intronic sequences of the *Ivr2A* soluble invertase markedly enhanced transcription of this gene.

Overall, the work presented here extends our understanding of the roles and regulation of soluble invertases in several significant ways. These progress from

interpretation of cell-level gene expression in a physiological context to analysis of transcriptional contributions by 5' promoter and intronic sequences

1. Spatial patterns of *Ivr2* mRNA expression changed in response to developmental signals and perturbation of sugar or cytokinin availability. For developing ovules, the timing and sites of this expression was compatible with rapid responses by maternal nutritive tissues that play key roles in early establishment of sink strength and kernel set. For root tips, sites of expression shifted from those favoring elongation and widespread import (with abundant sugars) to those favoring conservation of C resources and/or minimal elongation (with limited sugars or kinetin addition).
2. Expression of soluble invertases at both the mRNA and enzyme activity levels corresponded to carbohydrate-based differences in cell enlargement and root tip elongation, but not when growth was perturbed by other means.
3. Molecular analysis of genomic sequences revealed unusual features of the *Ivr2* invertases such as intact consensus sequences (otherwise split), very high GC content at the 5' end, and a number of cores for putative sugar and ABA response elements.
4. Analysis of 5' promoter and intron contributions to gene expression showed that upstream regions of both *Ivr1A* and *Ivr2A* were cytokinin and sugar responsive, and further, that the *Ivr2A* intron also contributed to this regulation.

Perhaps even more important is the contribution these studies make to future research in this direction. Subsequent work is already building rapidly on data presented here, which has allowed identification of the most valuable invertase genes, promoters, and introns for use in transgenic programs to improve maize yields. Toward this end, transgenic maize plants have been generated (in conjunction with Pioneer Hi Bred International, Johnston Iowa) using the *Ivr2A* 5' promoter and two of the upstream *Ivr2A* introns. Subsequent analysis of responses under field and greenhouse conditions will test the capacity of molecular materials generated and studied in the present work to direct advantageous changes in gene expression in transgenic maize. Results of the present studies also indicate that the coding sequence of the soluble *Ivr2A* invertase may be a key player in early kernel set and a strong candidate for targeted up-regulation in yield-improvement strategies.

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BIOGRAPHICAL SKETCH

Yong Wu was born on April 30 1963, in Chengdu, Sichuan Province PR China. He entered Peking University in September 1980 and earned his Bachelor of Science degree in plant biochemistry and plant biology in July 1984 Yong Wu started his graduate study at the Shanghai Institute of Plant Physiology, Academia Sinica, in September 1984 where he received his Master of Science degree in plant physiology in August 1987

After six years of experience in Sichuan University as research scientist and lecturer, Yong Wu attended a two-year advanced training course in plant biotechnology at the University of Pisa in Italy from 1993 to 1995

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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